REMARKS

Claims 1-3, and 5, 8-13, and 16-20 are pending and stand rejected, while claim 4 has been canceled and claims 6, 7, 14 and 15 have been withdrawn from consideration. Claims 1, 8, 9, 10 and 20 have been amended with this response. These claim amendments add no new matter. Support for the amendment to claims 1 and 8 can be found throughout the application, for example at page 14, lines 11-15. Support for the amendment to claims 9 and 10 can be found throughout the application, for example as antecedent basis in original independent claim 8 at page 47, line 11.

Obviousness-Type Double Patenting

The Office Action states that claims 18 and 19 remain rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-7 and 15-22 of U.S. Patent 6,372,427. In particular, the Office Action states that Applicants' traversal of this rejection in their last response relies upon an improper presumption that only those teachings in the specification necessary to serve as a "dictionary" for the terms used in the "prior art" claim may be considered in support of such a rejection. The Office Action urges that such a limited use of the teachings in an obviousness-type double patenting rejection is erroneous because "[c]ontrary to Applicants' assertions, the MPEP refers to two exceptions to the general prohibition of using the disclosure of a potentially conflicting patent or application in an ODP-Obviousness analysis" (emphasis added). In particular, the Office Action relies on the MPEP's interpretation of *In re Vogel* (422 F.2d 438 (CCPA 1970) for the proposition that "those portions of the specification which provide support for the patent claims may also be examined and considered when addressing the issue of whether a claim in the application defines an obvious variation of an invention claimed in the patent" (emphasis added, MPEP §804 II. B.1). Applicants respectfully continue to traverse this rejection for the reasons that follow.

While the cited excerpt from the MPEP may appear to provide a basis for using portions of Applicants' previous patent (U.S. Patent No. 6,372,427) as more than a mere dictionary for defining the meaning of terms in their corresponding "prior art claims," a true understanding of

the ruling in *In re Vogel* requires both a first-hand review of the actual decision as well as a review of its subsequent application by the Federal Circuit.

In particular, the ruling in *Vogel* (Attached hereto as Exhibit A) addresses the difficulty, in view of the fact that a claim defines a legal boundary and not a single physical reality, of trying to determine "what is or is not an obvious variation...(because)...[h]ow can it be obvious or not obvious to modify a legal boundary?" (*In re Vogel* 422 F.2d 438, at 442) The court's answer to this troublesome question imposed by the legal analysis required in obviousness-type double patenting, was that "the disclosure, however, sets forth at least one tangible embodiment within the claim, and it is less difficult and more meaningful to judge whether that thing has been modified in an obvious manner" (emphases added). Thus, the ruling in *Vogel* does not call for unrestrained application of all possible portions of the specification that might be argued to support a claim, but rather permits the use of "tangible embodiments" (*e.g.*, specific examples within the scope of each claim) so that obviousness might be judged relative to something corporeal, rather than relative to the legal abstraction of claim terminology.

Application of the ruling in *Vogel* to the instant facts would not lead to a finding that pending claims 18 and 19, drawn to pharmaceutical formulations that include "a pharmaceutically acceptable carrier," would be obvious in view of claims 1-7 and 15-22 of U.S. Patent 6,372,427, alone or in conjunction with the tangible HIV-1/MT-2 *in vitro* example (col. 17) that supports them. Indeed, as stated in their last response, Applicants have provided numerous supporting uses of the claimed compositions that are neither pharmaceutical in nature, nor specifically require a pharmaceutically acceptable carrier. Accordingly, the use of Applicants' teachings that "[t]he synthetic oligonucleotides of the invention may be used as a pharmaceutical composition when combined with a pharmaceutically acceptable carrier," is an overextension of the permissible scope of inquiry into support provided by the specification. This overextension is neither generally permissible in an obviousness-type double patenting rejection, nor specifically sanctioned by the ruling in *Vogel.*

Indeed, subsequent judicial rulings in this area have recognized the importance of limiting the indiscriminate use of portions of the specification, beyond those required for support/validity of the "prior art patent claims". For example, the court in *In re Kaplan* (789

F.2d 1574 (Fed. Cir. 1986) (Attached hereto as Exhibit B) warned against the unrestrained use of parts of the specification that "provide some of the support for (a term in the claim)", stating "[t]here is adequate support for (the term in the claim elsewhere)" (emphasis added). The Court went on to warn "[t]here is no way the board could have found appellants' claimed invention to be an obvious variation of what Kaplan claims except by treating the Kaplan patent disclosure as though it were prior art...(but)....[t]his has repeatedly been held in our precedents to be impermissible." (*In re Kaplan* 789 F. 2d 1574, at 1580).

Similarly, in the instant case the use of the cited "pharmaceutical" teachings is an improper overextension of the permissible use of the specification in support of an obviousness-type double patenting rejection, because the cited "pharmaceutical" teachings are by no means the single, essential and tangible "support" for the relevant "prior art claims" of Applicants' U.S. Patent No. 6,372,427. The use of these "pharmaceutical" teachings is, therefore, sanctioned neither by the court in *Vogel*, nor in the subsequent application of the *Vogel* decision by the Federal Circuit (*e.g.*, in *Kaplan*).

Reconsideration and withdrawal of this rejection is therefore respectfully requested.

Rejection under 35 U.S.C. §103

The Office Action states that claims 1-3, 5, 8-13, and 17 remain rejected under 35 U.S.C. §103(a) as being unpatentable over Gryaznov *et al.* (U.S. Patent No. 5,571,903) (the '903 patent) in view of Agrawal *et al.* (U.S. Patent No. 5,691,316) (the '316 patent). In addition, newly added claim 20 has been rejected under 35 U.S.C. §103(a) in view of the same combination of references. In support of this rejection, the Office Action states that, although "Applicants assert that the invention requires the presence of highly specific high affinity binding partners which enable the oligonucleotide to form a complex independent of, and prior to, annealing to target nucleic acid," such features are "not recited in the rejected claim(s)", and therefore "are not read into the claims". Applicants respectfully traverse this rejection for the reasons that follow.

First, Applicants note that the properties referred to are inherent aspects of the claimed cyclodextrin /adamantane and streptavidin / biotin oligonucleotide binding pairs, and such inherent properties are properly considered in distinguishing over art under §103. Indeed, one of the Graham Factual Inquiries for determining obviousness is "ascertaining the differences between the prior art and the claims in issue" (MPEP §2141), and, in doing so, the claimed invention must be considered as a whole (MPEP §2141.02). The MPEP further instructs that, in making this determination "we look not only to the subject matter which is literally recited in the claim in question...but also to those properties of the subject matter which are inherent in the subject matter" (emphasis added, at MPEP §2141.02). Accordingly, a complete analysis of whether the invention as a whole would have been obvious under 35 U.S.C. §103 must include a consideration of inherent properties of the claimed invention.

In the instant case, the claimed invention is drawn to paired oligonucleotides, complementary to a target nucleic acid, which are linked to either cyclodextrin and admantane or streptavidin and biotin, such that either of these high-affinity specific binding partners are bound together when the paired oligonucleotides are hybridized to tandem, non-overlapping complementary regions of the target nucleic acid. Applicants respectfully note that it is well known in the art that streptavidin/biotin and cyclodextrin/adamantine are inherently high-affinity binding pairs. In support, Applicants provide Exhibit C, (Weber *et al.* (1992) J. Am. Chem. Soc. 114: 3197-3200), which states that "[s]treptavidin is a tetrameric protein....that binds biotin with exceptionally high affinity ($K_a = 10^{13}$ M^{-1})" (at page 3197, 2nd paragraph of Introduction). Exhibit C thus provides evidence that the high affinity and specificity of the streptavidin /biotin interaction were well known in the art at the time of the invention. Similarly, Exhibit D (Harries *et al.* (2005) J. Am. Chem. Soc. 127: 2184-90) evidences the inherently high-affinity specific interaction between adamantine and cyclodextrin ("the association constants for the inclusion complex are typically 10^3-10^5 M^{-1}" (at page 2184, 1st paragraph of Introduction)).

These inherent properties of the claimed invention are in contrast to the low-affinity, non-specific hydrophobic interactions taught by the '903 patent. In particular, the '903 patent teaches "hydrophobic complexes" of, *e.g.*, lipophilic groups, including alkyl groups, fatty acids, fatty alcohols, steroids, waxes, fat-soluble vitamins, and the like," amongst others (see col. 6, lines 1-

31). These low-affinity, non-specific complexes described in the '903 patent, taken as a whole, alone or in combination with the '316 patent, would not lead one of skill in the art to the claimed invention of oligonucleotides having high-affinity, specific binding pairs. Accordingly, the claimed invention is not obvious in view of the '903 patent.

Furthermore, the Office Action states that Applicants have similarly improperly argued that "the '316 patent does not teach the covalent attachment of cyclodextrin to an oligonucleotide, which is a critical feature of one of the embodiments of the instant invention," but that "again, as stated above, the instant claims do not require the covalent attachment of cyclodextin to either of the first or second synthetic oligonucleotides of the claimed invention" (emphases added).

However, Applicants believe that both the language of the claim alone, and that language read in light of the specification, unambiguously does require the covalent attachment of the binding pair members to the oligonucleotide binding pairs. In particular, the claim requires that the "first oligonucleotide is linked to a first binding partner" and that the "second oligonucleotide is linked to a second binding partner." In further clarifying the covalent nature of the claimed "linked" groups, the specification teaches "[a]ny pair of moieties that can interact with each other non-covalently and which can be linked to oligonucleotides through covalent linkages can act as binding partners" (emphasis added, at page 14, lines 11-15). Furthermore, both Figure 1D and the Examples, beginning at page 40, support such a conclusion. Accordingly, Applicants assert that the invention, as claimed, is inherently distinct from the noncovalent cyclodextrin complexes taught by the '316 patent.

Nevertheless, in an effort to facilitate prosecution, and not in acquiescence to this rejection, Applicants have amended the relevant independent claims, 1 and 8, to require that each oligonucleotide be covalently linked to its binding partner. The claim as amended still further distinguishes over the '903 patent, when that reference is viewed alone as a whole, or in combination with the other references cited in the rejection.

Furthermore, in addition to these defects in the teachings of the '903 patent, there would have been be no motivation at the time of the invention to combine the teaching of the '903

patent with the teachings of Agrawal *et al.* (U.S. Patent No. 5,691,316) (the '316 patent) because there was no indication of a functional equivalency between the inherently low affinity, non-specific binding moieties taught by the '903 patent and the high-affinity cyclodextrin / adamantane binding pair disclosed in the '316 patent. Accordingly, there would have been no reasonable expectation of success of combining these references to arrive at adamantane/cyclodextrin- coupled oligonucleotide pairs with "increased cellular uptake...(and therefore) increase(d)...efficacy," as argued by the Examiner. This is so because there was no indication in the art that either the improved hybridization of the oligonucleotide pairs taught by the '903 patent or the improved cellular uptake of oligonucleotides in the presence of noncovalently linked cyclodextrin or adamantane/cyclodextrin complexes, as taught in the '316 patent, would be maintained if the distinct compositions taught be each were combined and appropriately substituted. Still further, Applicants note that the cited references, taken together, still do not teach or suggest the biotin/streptavidin binding pair of the rejected claims. Accordingly, reconsideration and withdrawal of the rejection is respectfully requested.

Rejection under 35 U.S.C. §112 (second paragraph)

The Office Action states that claims 9-10, and 20 have been rejected under 35 U.S.C. §112 (second paragraph) as being vague and indefinite for failing to particularly point out and distinctly claim the invention.

In particular, the Office Action states that claims 9-10 recite the limitation "duplex structure" in the first line, but that they depend from claim 8, which recites the phrase 'dimeric structure,' and does (not) recite the phrase "duplex structure."

Accordingly, claims 9 and 10 have been amended with this response to recite the phrase "dimeric structure," which has antecedent basis in claim 8, from which the amended claims depend. This amendment adds no new matter and completely addresses the issue of antecedent basis cited in this rejection.

The Office Action additionally states that, in claim 20, "the terms "phosphoramidates" and "phosphate esters" are repeated twice in the recited group. Applicants gratefully acknowledge the Examiner's recognition of their error in repeating the term "phosphate esters" twice within the recited group of claim 20. Applicants have corrected this error by appropriate amendment in this response. However, Applicants' supposed repetition of the term "phosphoramidates" in the same claim appears to be in reference to two, chemically-distinct claimed linkages, *i.e.*, phosphoramidates and phosphoramidites (emphases added). Both phosphoramidates and phosphoramidites are supported in the original application as filed (at page 17, lines 7 and 8), and are recognized in the art as distinct chemical structures. Amendment is therefore not needed in this particular instance.

Accordingly, reconsideration and withdrawal of the rejection, in view of the amendments and argument presented above, is respectfully requested.

Rejection under 35 U.S.C. §112 (first paragraph)

The Office Action states that claims 16-19 stand rejected under 35 U.S.C. § 112, first paragraph because "the specification, while being enabling for using the claimed pharmaceutical formulation and compositions *in vitro*, does not reasonably provide enablement for the *in vivo* use of the claimed formulation or compositions for treatment purposes." Applicants respectfully traverse this rejection for the reasons that follow.

As an initial matter, Applicants respectfully note that enablement of the rejected claims has been acknowledged in the instant rejection. In particular, the Office Action states that "while being enabling for using the claimed pharmaceutical formulations and compositions *in vitro*" (Office Action, at page 7). Applicants respectfully note that the rejected claims are composition claims, not method of use claims, and therefore the appropriate enablement inquiry addresses whether the Applicant has enabled the public to make the full scope of the claimed compositions, but requires that only a single use of the claimed composition be taught. The MPEP (at 2164.01(c)) states that "when a compound or composition claim is not limited by a recited use" (as in this case), then "any enabled use...is sufficient to preclude a rejection for nonenablement

based on how to use." The same section of the MPEP goes on to state "if any use is enabled when multiple uses are disclosed, the application is enabling for the claimed invention" (emphasis added). As the Office Action acknowledges that *in vitro* uses of the claimed pharmaceutical formulations have been enabled, the "use" of the claimed compositions is therefore necessarily supported to the extent required by law under 35 U.S.C. §112. Reconsideration and withdrawal of the rejection is therefore respectfully requested.

Furthermore, beyond this clear legal basis for enablement of the claimed pharmaceutical formulations, Applicants respectfully note for the record the following matters relating to this renewed enablement rejection.

The Office Action states that Applicants' previously-presented argument that "the supporting references in this rejection addressed only the use of monomeric antisense oligonucleotides and are not directly relevant to the instant invention," is not persuasive because "the enablement of the instant invention is further complicated by the need to deliver multiple oligonucleotides to a single target within a cell" (Office Action, at pages 7-8). However, Applicants respectfully note that this supposed "complication" is mere conjecture and opinion. Indeed, relevant to enablement of rejected claims 16 and 17, the Office Action earlier recognized Applicants' own U.S. Patent No. 5,691,316 as teaching that "cyclodextrin or admantine/cyclodextrin modifications are known in the prior (art) to increase the cellular uptake of oligonucleotides comprising these modifications and thereby increase the efficacy of their application and reduce the dose required (Office Action at page 5-6, citing Agrawal *et al.* (U.S. Patent No. 5,691,316)). Therefore, while the instant Office Action has heavily weighted the teachings of Applicants' own patent against the claims in the instant application in an obviousness rejection, it has not even considered these same teachings in assessing the reasonable enablement of the claimed invention as a whole.

Applicants maintain that, for those reasons stated above as well as those provided in their last response, the claimed invention, as a whole, is enabling to a person of skill in art without undue experimentation. Furthermore, Applicants respectfully assert that the last Office Action, in stating that the instant specification is "enabling for using the claimed pharmaceutical formulations and compositions *in vitro*," has implicitly acknowledged enablement of the claimed

pharmaceutical formulations. Accordingly, reconsideration and withdrawal of the rejection is respectfully requested.

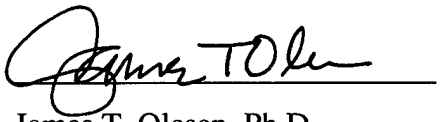
CONCLUSION

In view of the foregoing remarks, Applicants respectfully submit that the pending claims are in condition for allowance. If a telephone interview would advance prosecution of the application, the Examiner is invited to call the undersigned at the number listed below.

A Petition for a Two (2) Month Extension of Time, and authorization of payment of the corresponding fee accompanies this Response. Please charge any additional fees or refund any overpayment to Deposit Account No. 08-0219.

Respectfully submitted,

Date: June 2, 2005



James T. Olesen, Ph.D.
Attorney for Applicant
Reg. No.: 46,967

WILMER CUTLER PICKERING HALE AND DORR LLP
60 State Street
Boston, MA 02109
Tel: (617) 526-6000
Fax: (617) 526-5000

57 C.C.P.A. 920, *; 422 F.2d 438, **;
1970 CCPA LEXIS 423, ***; 164 U.S.P.Q. (BNA) 619

LEXSEE 422 F.2D 438

IN RE VIRGIL W. VOGEL AND PAUL W. VOGEL

No. 8198

United States Court of Customs and Patent Appeals

57 C.C.P.A. 920; 422 F.2d 438; 1970 CCPA LEXIS 423; 164 U.S.P.Q. (BNA) 619

Oral argument November 5, 1969

March 5, 1970 *

*** Petition for rehearing denied June 11, 1970.**

PRIOR HISTORY: [***1]

APPEAL from Patent Office, Serial No. 338,158

DISPOSITION:

Modified.

LexisNexis(R) Headnotes

COUNSEL:

Harvey B. Jacobson, Jacob Shuster, attorneys of record, for appellants.

Joseph Schimmel for the Commissioner of Patents. *Fred W. Sherling,* of counsel.

OPINIONBY:

LANE

OPINION: [**439]

[*921] Before RICH, ALMOND, BALDWIN, LANE, Associate Judges, and MATTHEWS, Judge, sitting by designation.

[*922] LANE, Judge, delivered the opinion of the court:

This appeal is from the decision of the Patent Office Board of Appeals affirming the rejection of all claims (7, 10 and 11) in appellants' patent application serial No. 338,158, filed January 16, 1964, for "Process of Preparing Packaged Meat Products for Prolonged Storage."

The ground of rejection for each claim is double patenting, based upon the claims of appellants' U.S. patent *3,124,462*, issued March 10, 1964, in view of a reference patent to Ellies, Re. 24,992, reissued May 30, 1961.No terminal disclaimer has been filed.

The Appealed Claims

Claims 7 and 10 are directed to a process of packaging meat generally. Claim 10 is illustrative:

10. A method for prolonging the storage life of packaged meat products comprising the steps of: [***2] removing [**440] meat from a freshly slaughtered carcass at substantially the body bleeding temperature thereof under ambient temperature conditions; comminuting the meat during an exposure period following slaughter while the meat is at a temperature between said bleeding and ambient temperatures; sealing the comminuted meat within a flexible packaging material having an oxygen permeability ranging from 0.01 X 10(-10) to 0.1 X 10(-10) cc.-mm/sec/cm(2)/cm Hg at 30 degrees C. during said exposure period and before the meat has declined in temperature to the ambient temperature; and rapidly reducing the

temperature of the packaged meat to a storage temperature below the ambient temperature immediately following said packaging of the meat.

The invention is based on appellants' discovery that spoilage and discoloration of meat are markedly accelerated if the meat is allowed to reach ambient temperature before packaging.

Claim 11 is directed to a similar process specifically limited to beef.

Prior Art

The only reference of record is Ellies. Ellies teaches the use of meatpackaging material having the oxygen permeability range recited in the claims.

The Patent

Appellants' [***3] patent, which is not prior art, claims a method of processing pork. Claim 1 of the patent is illustrative.

1. A method of preparing pork products, comprising the steps of: boning a freshly slaughtered carcass while still hot into trimmings; grinding desired carcass trimming while still warm and fluent; mixing the ground trimmings while fluent and above approximately 80 degrees F., mixing to be completed not more than approximately 3 1/2 hours after the carcass has been bled and stuffing the warm and fluent mixed trimmings into air impermeable casings.

[*923] The board characterized the rejection as follows:

The sole ground of rejection is that claims 7, 10 and 11 are unpatentable over appellants' copending patented claims in Vogel et al., in view of Ellies. This is a double-patenting type rejection, whose statutory basis is *35 U.S.C. 101,* as indicated in *In re Ockert, 44 CCPA 1024;* 1957 *CD 404; 722 OG 222; 245 F.2d 467; 114 USPQ 330.*

Thus the board viewed this case as involving "same invention" type double patenting. The board then discussed the differences between the appealed claims and the patent claims and found that the former did not define a "patentable advance" [***4] over the latter. It is thus clear that the board was not at all dealing with "same invention" type double patenting but with the "obvious variation" type. n1 The board found that the appealed claims merely extended the pork process to beef, and that this was not a "patentable advance." Such language states only a conclusion, since patentability is the very issue to be determined. The board gave the following analysis to support its conclusion:

n1 [1] The examiner's final rejection and the solicitor's oral argument contend that only a single invention is involved. They go on to point out the differences. Apparently they were thinking that "invention" means "patentable invention." This has not been the language of the law since January 1, 1953. See *35 USC 102,* 103.

We agree with the Examiner's reasons for holding the application of the claimed method to beef to be an unpatentable adaptation. In addition, the definition of "sausage" in Webster's 3rd New International Dictionary of 1963, on page 2019 is pertinent:

"sausage - a highly seasoned finely divided meat that is usually a [**441] mixture (as of beef or pork) * * *"

The examiner's reasons as stated in his [***5] answer were that the process steps are essentially the same, and the choice of beef rather than pork "is of no patentable significance since this would appear to be a judicious choice of available meat products, well within the ordinary skill of the art, and particularly so, in the absence of any unusual or unobvious result."

The board's use of the dictionary meaning of "sausage," as above quoted, is apparently intended to show that beef and pork are equivalents. Whatever may be their equivalency in other contexts, the dictionary definition of "sausage" does not show that beef and pork are equivalents in the sense of the invention now claimed. Appellants contend that the examiner and the board used the disclosure of the patent as a basis for concluding obviousness. To the effect that consideration of the patent disclosure is improper in testing for obvious-type double patenting, appellants cite *In re Baird, 52 CCPA 1747, 348 F.2d 974, 146 USPQ 579 (1965).*

[*924] The proceedings below in this case indicate the advisability of a restatement of the law of double patenting as enunciated by this court.

[2] The first question in the analysis is: Is the same invention being [***6] claimed twice? *35 USC 101* prevents two patents from issuing on the same invention. See, e.g., *In re Boylan, 55 CCPA 1041, 392 F.2d 1017, 157 USPQ 370 (1968).*As we have said many times, "invention" here means what is defined by the claims, whether new or old, obvious or unobvious; it must not be used in the ancient sense of "patentable invention," or hopeless confusion will ensue. By "same invention" we mean identical subject matter. Thus the invention defined by a claim reciting "halogen" is not the same as that defined by a claim reciting "chlorine," because the former is broader than the latter. On the other hand, claims may be differently worded and still define the same invention. Thus a claim reciting a length of "thirty-

six inches" defines the same invention as a claim reciting a length of "three feet," if all other limitations are identical. [3] In determining the meaning of a word in a claim, the specification may be examined. It must be borne in mind, however, especially in non-chemical cases, that the words in a claim are generally not limited in their meaning by what is shown in the disclosure. Occasionally the disclosure will serve as a dictionary for terms appearing [***7] in the claims, and in such instances the disclosure may be used in interpreting the coverage of the claim. *In re Baird, supra.* A good test, and probably the only objective test, for "same invention," is whether one of the claims could be literally infringed without literally infringing the other. If it could be, the claims do not define identically the same invention. Thus is essentially the test applied in *In re Eckel, 55 CCPA 1068, 393 F.2d 848, 157 USPQ 415 (1968).* There the court rejected the idea of "colorable variation" as a comparison category and stated that inventions were either the same, or obvious variations, or unobvious variations. The court's holding in Eckel was that same invention means identically same invention.

[4] If it is determined that the same invention is being claimed twice, *35 USC 101* forbids the grant of the second patent, regardless of the presence or absence of a terminal disclaimer. If the same invention is not being claimed twice, a second question must be asked.

The second analysis question is: Does any claim in the application define merely an obvious variation of an invention disclosed and claimed in the patent? In considering the question, [***8] the patent disclosure may not be used as prior art. *In re Boylan, supra; In re Aldrich, 55 CCPA 1431, 398 F.2d 855, 158 USPQ 311 (1968).* This does not mean that the disclosure may not be used at all. As pointed out above, in certain instances it may be used as a dictionary to learn [*925] the meaning of terms in a claim. It may [**442] also be used as required to answer the second analysis question above. We recognize that it is most difficult, if not meaningless, to try to say what is or is not an obvious variation of a claim. [5] A claim is a group of words defining only the boundary of the patent monopoly. It may not describe any physical thing and indeed may encompass physical things not yet dreamed of. How can it be obvious or not obvious to modify a legal boundary? The disclosure, however, sets forth at least one tangible embodiment within the claim, and it is less difficult and more meaningful to judge whether that thing has been modified in an obvious manner. It must be noted that this use of the disclosure is not in contravention of the cases forbidding its use as prior art, nor is it applying the patent as a reference under *35 USC 103,* since only the disclosure [***9] of the invention claimed in the patent may be examined.

If the answer to the second question is no, there is no double patenting involved and no terminal disclaimer need be filed. If the answer is yes, a terminal disclaimer is required to prevent undue timewise extension of monopoly.

We now apply this analysis to the case before us.

The first question is: Is the same invention being claimed twice? The answer is no. The patent claims are limited to pork. Appealed claims 7 and 10 are limited to meat, which is not the same thing. Claims 7 and 10 could be infringed by many processes which would not infringe any of the patent claims. Claim 11 is limited to beef.Beef is not the same thing as pork.

We move to the second question: Does any appealed claim define merely an obvious variation of an invention disclosed and claimed in the patent? We must analyze the claims separately.

As to claim 11 the answer is no. This claim defines a process to be performed with beef. We must now determine how much of the patent disclosure pertains to the invention claimed in the patent, which is a process to be performed with pork, to which all the patent claims are limited. The specification [***10] begins with certain broad assertions about meat sausages. These assertions do not support the patent claims. The patent claims recite "pork" and "pork" does not read on "meat." To consider these broad assertions would be using the patent as prior art, which it is not. The specification then states how the process is to be carried out with pork. This portion of the specification supports the patent claims and may be considered. It describes in tabular form the time and temperature limits associated with the pork process. Appealed claim 11, reciting beef, does not read on the pork process disclosed and claimed in the patent. Further, we conclude that claim 11 does not define merely an obvious variation of the pork process. The specific [*926] time and temperature considerations with respect to pork might not be applicable to beef. There is nothing in the record to indicate that the spoliation characteristics of the two meats are similar. Accordingly, claim 11 does not present any kind of double patenting situation.

Appealed claim 10, supra, will now be considered. It recites a process to be performed with "meat." "Meat" reads literally on pork. The only limitation [***11] appearing in claim 10 which is not disclosed in the available portion of the patent disclosure is the permeability range of the packaging material; but this is merely an obvious variation as shown by Ellies. The answer to the second analysis question, therefore, is yes, and the claim is not allowable in the absence of a terminal disclaimer. The correctness of this conclusion is demonstrated by observing that claim 10, by reciting

"meat," includes pork. Its allowance for a full term would therefore extend the time of monopoly as to the pork process. It is further noted that viewing the inventions in reverse order, i.e. as though the broader claims issued first, does not reveal that the narrower [**443] (pork) process is in any way unobvious over the broader (meat) invention disclosed and claimed in the instant application. The same considerations and result apply to claim 7.

The decision of the board is affirmed as to claims 7 and 10 and reversed as to claim 11.

LEXSEE 789 F.2D 1574

IN re LEONARD KAPLAN and WELLINGTON EPLER WALKER

No. 85-2522

UNITED STATES COURT OF APPEALS FOR THE FEDERAL CIRCUIT

789 F.2d 1574; 1986 U.S. App. LEXIS 21209; 229 U.S.P.Q. (BNA) 678

May 6, 1986

PRIOR HISTORY: [**1]

Appealed from: U.S. Patent and Trademark Office Board of Patent Appeals and Interferences.

LexisNexis(R) Headnotes

COUNSEL:

Steven T. Trinker; of Danbury, Connecticut, Argued, for Appellant. On the brief was Norman L. Balmer, Law Department, Union Carbide Corp., of Danbury, Connecticut.

Harris A. Pitlick, Associate Solicitor, of Arlington, Virginia, Argued, for Appellee U.S. Patent and Trademark Office. With him on the brief were Joseph F. Nakamura, Solicitor and Fred E. McKelvey, Deputy Solicitor.

JUDGES:

Rich, Circuit Judge, Nichols, Senior Circuit Judge, and Nies, Circuit Judge.

OPINIONBY:

RICH

OPINION:

[*1574] RICH, Circuit Judge.

This appeal is from the decision of the United States Patent and Trademark Office (PTO) Board of Patent Appeals and Interferences (board) rejecting, under 37 CFR 1.196(b), the single claim of appellants' application serial No. 364,221, filed April 1, 1982, entitled "Homogeneous Liquid Phase Process for Making Alkane Polyols," on the sole ground of "double patenting, because it constitutes an improper extension of monopoly for an invention claimed by Kaplan." We reverse.

Background

The Kaplan and Walker application at bar and the cited Kaplan patent, No. 3,944,588, [**2] issued Mar. 16, 1976, to one of the appellants on an application filed Jan. 2, 1975, are both assigned to Union Carbide Corporation, the real party in interest. As is apparent, the Kaplan patent application was pending only about fourteen and a half months. It was copending with the great-great-grandparent of the application at bar, filed Sept. 30, 1975. Its title is "Catalytic Process for Polyhydric Alcohols and Derivatives." The Kaplan patent contains one independent claim and thirteen dependent [*1575] claims. The claims most relevant here are those incorporated in dependent claim 4, which is the only claim specifically relied on by the board to support its double patenting rejection. They read as follows (emphasis ours):

> 1. The process of making alkane diols and triols having from 2 to 3 carbon atoms in the molecule which comprises reacting in a homogeneous liquid phase mixture of hydrogen and oxides of carbon in the presence of a rhodium carbonyl complex and a trialkanolamine borate at a pressure of from about 1000 psia to about 50,000 psia correlated with a temperature of about 100 degrees C to about 375 degrees C sufficient to produce said diols and triols. [**3]

> 2. The process of *claim 1* wherein the temperature is from about 150 degrees C to about 300 degrees C.
>
> 4. The process of *claim 2* wherein the reaction is effected *in the presence of an organic solvent.*

Among organic solvents disclosed and specifically claimed in the Kaplan patent are two known as "tetraglyme" (in more explicit nomenclature, dimethyl ether of tetraethylene glycol) and sulfolane. Two of the Kaplan dependent claims (10 and 11) individually name these specific solvents, respectively. No claim in Kaplan calls for a solvent *mixture*, which is significant with respect to the double patenting rejection for reasons which will appear. There are, however, a number of *examples* of mixed solvents in Table VI of the Kaplan patent specification, particularly Example 45, upon which the board relied. Example 45 is specific to a mixture of "Tetraglyme/sulfolane (65/10)." The heading of Table VI is "Triisopropanolamine Borate in Mixed Solvents."

Against this much of the background, we now reproduce the single claim on appeal of this *joint* application of Kaplan and Walker which stands rejected for double patenting in view of claim 4 of the Kaplan [**4] patent (emphasis ours):

> *In the* homogeneous liquid phase *process* of producing alkane polyols by the reaction of oxides of carbon and hydrogen in the presence of a rhodium catalyst in which rhodium is complexed with carbon monoxide to provide a rhodium carbonyl complex at a temperature between about 100 degrees C. to about 375 degrees C. and a pressure between about 1000 psia to about 50,000 psia, *the improvement* which comprises effecting said reaction *in a solvent mixture of tetraglyme and sulfolane* under conditions whereby such solvent mixture is essentially inert and the *rate of formation of such alkane polyol is greater* than would be obtained by effecting said reaction under equal conditions using *tetraglyme or sulfolane* as the solvent.

It will be observed from a comparison of this claim with the Kaplan claims reproduced above that the Kaplan and Walker (joint) claim at bar is, generally speaking, defined as an improvement on the Kaplan (sole) catalytic process of producing alkane polyols (diols and triols) by reacting hydrogen and carbon oxides (e.g., carbon monoxide) in an organic solvent. The reason why the process using the solvent *mixture* [**5] of the appealed claim was not *claimed* in the Kaplan patent, although it is *disclosed* in the patent specification, is that Kaplan alone was not the inventor of that process; it was the *joint* invention of Kaplan and Walker and therefore the application on appeal was filed. The reason it was *disclosed* in Kaplan's patent was that it was part of the "best mode" of practicing Kaplan's catalytic process. *See 35 USC § 112*, first paragraph. ("The specification . . . shall set forth the best mode contemplated by the inventor of carrying out his invention.") By the time Kaplan filed his application he knew of ("contemplated") the Kaplan and Walker improvement on his own sole invention and therefore he disclosed it. It is a given, of course, that a sole inventor and joint inventors including the sole inventor are separate "legal entities," a legal proposition from which certain legal consequences flow, *In re Land and Rogers, 54 C.C.P.A. 806, 368 F.2d 866, 879, 151 U.S.P.Q. (BNA) 621, 633 (CCPA 1966)*, n1 "such as who must [*1576] apply [**6] for patent." It is worth remembering an axiomatic statement on the same page of the *Land and Rogers* case, which is also applicable here:

> When the joint and sole inventions are related, as they are here, inventor A commonly discloses the invention of A & B in the course of describing his sole invention and when he so describes the *invention* of A & B he is not disclosing "prior art" to the A & B invention, even if he has legal status as "another." [the reference to "another" is to that word as used in *35 USC 102*(e) and (g).]

n1 All applications involved in this case were filed and the Kaplan patent had issued before amendment of *35 USC § 116* by P.L. 98-622 of Nov. 8, 1984, sec. 104(a), 98 Stat. 3384, now paragraph one of *35 USC § 116*, which reads:

> When an invention is made by two or more persons jointly, they shall apply for patent jointly and each make the required oath, except as otherwise provided in this title. Inventors may apply for a patent jointly even though (1) they did not physically work

> together or at the same time, (2) each did not make the same type or amount of contribution, or (3) each did not make a contribution to the subject matter of every claim of the patent.

> The first sentence is the substance of the law at the times involved in this case. The second sentence is the liberalization added by the 1984 amendment, which, had it been available, might have obviated the problem in this case.

[**7]

Having been filed during the pendency of the Kaplan application, the present Kaplan and Walker application with its single mixture claim had a difficult time in the PTO resulting in the passage of much time. To summarize, there were two appeals to the board prior to the appeal which resulted in the decision now before us. Three continuation applications were filed under *37 CFR 1.60*. The last of these, filed Jan. 9, 1981, is the present application and was appealed to the board from the examiner's rejections under § § 102(g), 102(a), and 103 based on the Kaplan patent and a patent to Pruett. Declarations under *37 CFR 1.131* were filed by appellants and by Kaplan explaining who invented what and when.

The board reversed all of the examiner's grounds of rejection and entered its own rejection on the ground of double patenting, as stated in the first paragraph hereof, which rejection was adhered to on reconsideration. Applicants sought reconsideration by the board "rather than reopening prosecution as is permitted under 37 C.F.R. 1.196(b)." Appellants then took this appeal. Therefore, this is the first review of the board's new rejection, explicated in its two opinions.

To [**8] indicate the reasoning of the board, we quote most of the paragraph of its initial opinion in which it made its new rejection (all emphasis ours):

> Under the provisions of 37 CFR 1.196(b), we reject claim 1 [there is no other] on the ground of double patenting, because it constitutes *an improper extension of monopoly* for [sic, of] an invention claimed by Kaplan. . . . At least claim 4 of the Kaplan patent and appellant's claim 4 of the Kaplan patent and appellant's claim 1 *embrace* common subject matter. Both claims are generic n2 and both claims *would be infringed* by a process which utilized rhodium and trialkanolamine borate as the catalyst and a *mixed solvent* as the organic solvent. Example 45 of the Kaplan patent clearly shows that the term solvent, as used in Kaplan's claims is intended to *embrace* the mixed solvent of Example 45. Further, appellants' claim 1 is sufficiently broad to *encompass* the use of a trialkanolamine borate in conjunction with the rhodium catalyst. Because both claims *embrace the same subject matter*, allowance of the instant application would amount to "double patenting of the improper extension of monopoly type" * as [**9] termed by Judge Almond in *In re Thorington, 57 C.C.P.A. 759, 769, 418 F.2d 528, 537, 163 U.S.P.Q. (BNA) 644, 650 (CCPA 1969).*

* We prefer the term "improper extension of monopoly" rather than "obviousness type double patenting" because the improper extension of monopoly occurs as a result of the *same* [*1577] *subject matter being claimed.* The rejected claims [sic] before us may well be drafted so broadly as to also embrace subject matter which is unobvious over the Kaplan patent. Nevertheless, for all practical purposes, the rejected claims [sic] serve to extend the monopoly for that subject matter *embraced* by the claims which is the same as that falling within the *embrace* of the Kaplan claims.

> n2 Just what the board meant by saying "both claims are generic" is not clear to us. The claims speak for themselves. Kaplan's claim 4 defines the solvent used in the process, which is the limitation under discussion by the board, as "an organic solvent." Appellants' claim on appeal defines the solvent as "a solvent mixture of tetraglyme and sulfolane." Far from being "generic," the latter looks very much like a quite specific species of the genus "organic solvent."

[**10]

The board then discussed *In re Vogel, 57 C.C.P.A. 920, 422 F.2d 438, 164 U.S.P.Q. (BNA) 619 (CCPA 1970),* a case on which appellants as well as the PTO rely before us, and continued:

> Accordingly, the instant claim, which reads on subject matter disclosed in and *embraced* by the claims of the Kaplan patent, cannot be granted absent filing of a *terminal disclaimer* to prevent undue timewise extension of monopoly.

The imposition of the terminal disclaimer originated with the board in conjunction with its origination of the double patenting rejection. From the dates set forth above, it will be seen that its effect would be to cause any patent issuing on the application at bar to expire on March 16, 1993, the expiration date of the Kaplan patent, assuming a term of 17 years, so that it would have a term of less than 7 years. Appellants have refused that option. And, of course, if the board's claim analysis is correct, appellants would gain little or nothing from the patent because the invention of the appealed claim, using the mixed solvents, is already *covered* by the Kaplan patent until the date of its expiration.

Following the filing [**11] of what the board characterized as "appellants' well-drafted Request for Reconsideration," of some 20 pages, the board wrote its second opinion, discussing further *In re Vogel* and some other CCPA double patenting opinions, emphasizing the following:

> The Kaplan patent deliberately chose to claim the use of organic solvents as a vehicle for carrying out the claimed process. . . . The Kaplan patent discloses numerous solvents . . . five of which are solvent mixtures and one of which is the tetraglyme/sulfolane solvent mixture which is claimed by appellant as the essential feature of their process. Surely, the tetraglyme/sulfolane solvent of Table VI *provides some of the support* for the term "organic solvent" as used in claim 4 of the Kaplan patent. [Emphasis ours.]
>
> . . .
>
> As indicated by the patent, the term "solvent" includes the same mixed solvent claimed by appellants. Being in some aspects the same, the subject of appellants' claims [sic] would have been *prima facie* obvious from the subject matter of the claims in the Kaplan patent. Appellants' evidence of unexpected results teaches no more than that which is disclosed in the patent and which is properly [**12] considered supportive of the claims [sic], i.e., that mixed solvents give superior yields. Appellants' evidence does not overcome the *prima facie* case and a "terminal disclaimer" is necessary. . . .

OPINION

Double Patenting Generally

We reverse the board's double patenting rejection essentially for two reasons: (1) It has confused double patenting with "domination" which, by itself, does not give rise to "double patenting" and (2) it has used the disclosure of appellants' joint invention in the Kaplan patent specification as though it were prior art, which it is not, to support the obviousness aspect of the rejection.

By domination we refer, in accordance with established patent law terminology, to that phenomenon, which grows out of the fact that patents have claims, whereunder one patent has a broad or "generic" claim which "reads on" an invention defined by a narrower or more specific claim in another patent, the former "dominating" the latter because the more narrowly [**13] claimed invention cannot be practiced without infringing the broader claim. To use the words of which the board seemed to be enamored, the broader claim "embraces" or "encompasses" the subject matter defined by the narrower claim. In possibly simpler terms, one patent dominates another if a claim of the first patent reads on a device built or process practiced according to the second patent disclosure. This commonplace [*1578] situation is not, per se, double patenting as the board seemed to think. *In re Sarett, 51 C.C.P.A. 1180, 327 F.2d 1005, 1014, 1015, 140 U.S.P.Q. (BNA) 474, 482, 483 (CCPA 1964).* (See particularly the quotations from E. Stringham's *Double Patenting* (1933) about terms such as "covered" and "embraced.")

With respect to the board's concern about "extension of monopoly," the PTO Solicitor's brief, while supporting the board, properly deplores its use of the ambiguous word "monopoly," preferring to use the more accurate and less emotion-generating expression n3 "extension of patent rights," explaining this in a footnote reading:

> Both the board in this case and some prior decisions of the CCPA use the term "monopoly" in referring [**14] to the rights obtained through the grant of a patent. We prefer to refer to "patent rights" based on the rationale given by Chief Judge Markey in his article "Why Not the Statute?," *65 J. Pat. Off. Soc'y 331, 331-333 (1983).*

See also Carl Schenck, A.G. v. Nortron Corp., 713 F.2d 782, 218 U.S.P.Q. (BNA) 698, n.3 (Fed. Cir. 1983); Kayton on Patents, 2d ed., 1-27, "E. Patents: Property Versus Monopoly." *Compare, Robinson on Patents* (1890) Chapt. II § § 11-44.

> n3 The difficulty is that "monopoly" is used in different senses in patent and antitrust law, hence its ambiguity. Because of its antitrust connotations and association with illegality in connection therewith, it often evokes negative reactions inappropriate to a dispassionate analysis of patent law problems. *See American Hoist & Derrick Co. v. Sowa & Sons, Inc., 725 F.2d 1350, 1367, 220 U.S.P.Q. (BNA) 763, 776 (Fed. Cir. 1984).*

More to the point of the board's concern, however, one must [**15] inquire more closely than did the board: extension of what patent right? Any patent granted on the application at bar will have the single claim on appeal which is expressly limited to carrying out the Kaplan process using the specific solvent *mixture* of tetraglyme *and* sulfolane invented by appellants. Is this an extension of a patent on Kaplan's invention -- Kaplan who never conceived of using that mixture? When Kaplan's (sole) patent expires, and assuming appellants get their joint patent, the world will still be free to use (so far as these two patents go) the Kaplan process so long as appellants' solvent mixture is not used in it. Of course, it may be that everyone will want to use the improvement, but that is commonly the case when dominating patents expire with improvement patents still outstanding.

In further clarification of the distinction between domination and double patenting as currently understood, we repeat a passage from E. Stringham's *Double Patenting* at 207, previously quoted in *Sarett*:

> One of the simplest, clearest, soundest and most essential principles of patent law, is that a later invention may be validly patented, altho [sic] dominated [**16] by an earlier patent, whether to the same or to a different inventor. No one will seriously deny the correctness of this statement, in principle. But it is incessantly lost sight of when an actual case must be decided.

"May be validly patented" of course implies that the "later invention" at least complies with the requirements for patentability found in the statute, namely, novelty, utility, and unobviousness as established by evidence of prior art, which a description of the later invention is not. Domination is an irrelevant fact.

The development of the modern understanding of "double patenting" began in the Court of Customs and Patent Appeals (CCPA) about the time of *In re Zickendraht, 50 C.C.P.A. 1529, 319 F.2d 225, 138 U.S.P.Q. (BNA) 22 (CCPA 1963),* a rather unusual case in that there was no majority opinion because only two judges joined each of the two principal opinions. Neither *opinion* therein, therefore, can be regarded as controlling precedent in this court. That case is noteworthy primarily for the suggestion in a concurring opinion that the appellant might have disposed of the rejection by filing a terminal disclaimer under *35 USC § 253.* [**17] This suggestion precipitated a steady stream of appeals over the next few years dealing with double patenting and the effectiveness or otherwise of terminal disclaimers which resulted in revisions of the PTO's rules, [*1579] guidelines, and Manual of Patent Examining Procedure on the matter. By the time of *In re Vogel*, 1970, the court saw fit to make a restatement of the law of double patenting which serves as a good starting place for deciding this case.

The first question treated in the *Vogel* restatement is whether the *same invention* is being claimed twice. If so, *Vogel* states, *35 USC § 101* prevents two patents from issuing. *In re Boylan, 55 C.C.P.A. 1041, 392 F.2d 1017, 157 U.S.P.Q. (BNA) 370 (CCPA 1968).* We need not linger over this question as that is not the rejection made by the board here, notwithstanding what it said about what the claims "embrace." n4

> n4 For the latest decision of this court on the "same invention" double patenting issue see *Studiengesellschaft Kohle mb H v. Northern Petrochemical Co., 784 F.2d 351, 228 U.S.P.Q. (BNA) 837 (Fed. Cir. 1986),* a case in which it was expressly held that "obviousness-type double patenting is not involved in this case." The opinion may be of interest, however, for what it

has to say about "domination" and delay in the issuance of a second patent due to proceedings in the PTO. F.2d at , 228 U.S.P.Q. at 841.

[**18]

The second question, says *Vogel*, is: "Does any claim in the application define merely an obvious variation of an invention disclosed and claimed in the patent? In considering the question, the patent disclosure may not be used as prior art. *In re Boylan, supra; In re Aldrich, 55 C.C.P.A. 1431, 398 F.2d 855, 158 U.S.P.Q. (BNA) 311 (CCPA 1968).*" The opinion went on to describe, and resolve, some of the logical difficulties in reaching a decision on whether there is or is not what has come to be known consistently as "obviousness-type double patenting," the ground of rejection now before us.

Should there be any doubt about the true ground of rejection before us in view of the board's stated preference for the term "improper extension of monopoly" instead of "obviousness-type double patenting," we observe that the brief of the PTO Solicitor states the issue to be whether the board was correct in rejecting the claim "on the ground of double patenting of the obviousness type." In the summary of argument is the statement: "Claim 4 [of Kaplan] and its [**19] supporting disclosure render the subject matter of the appealed claim obvious." In the argument proper, the brief says the main disagreement between appellants and the board "appears to be the interpretation of the so-called 'second analysis question' discussed in *In re Vogel*," and that is the obviousness-type double patenting question, which we quoted above.

We will say a word about the board's desire to depart from the established terminology in the law of double patenting for the reason, quoted earlier, that "the improper extension of monopoly [i.e., of the patent right] occurs as a result of the same subject matter being claimed." The board's first opinion said it was adopting terminology from Judge Almond's opinion in *Thorington*, another CCPA case in which the court undertook to restate the law of double patenting. Reading Judge Almond's opinion will show that it recognizes two types of double patenting: same invention type and obviousness type, the main significance of the distinction being that filing a terminal disclaimer is permitted to cure an obviousness type situation but not a same invention type situation. (The fact that the board here demanded a terminal [**20] disclaimer as a condition for allowance of the claim is another indication of the true nature of its rejection.) It is also clear from Judge Almond's opinion that he was using "extension of monopoly type" as synonymous with obviousness type, merely as a way of distinguishing from same invention type double patenting.

The main reason why one cannot use "extension of monopoly" *as a type designation*, however, is that it cannot serve that purpose because the basis for *both* same invention and obviousness-type double patenting rejections is timewise extension of the patent right. All proper double patenting rejections, of either type, rest on the fact that a patent has been *issued* and later issuance of a second patent will continue protection, beyond the date of expiration [*1580] of the first patent, of the very same invention claimed therein (same invention type double patenting) or of a mere variation of that invention which would have been obvious to those of ordinary skill in the relevant art (obviousness-type double patenting). In the latter case, there must be some clear [**21] evidence to establish why the variation would have been obvious which can properly qualify as "prior art." Even if obviousness of the variation is predicated on the level of skill in the art, prior art evidence is needed to show what that level of skill was.

Obvious Variation of what Kaplan Claims

We turn now to consideration of the obviousness aspect of this obviousness-type double patenting rejection, which had to be based, of course, on what is *claimed* in the Kaplan patent. The board relied on Kaplan claim 4, which depends from claim 2, which depends from claim 1. These claims are set forth above. The board relied on the fact that claim 4 calls for "an organic solvent." The board did not say that the use of appellants' "solvent mixture of tetraglyme and sulfolane" would be obvious from claim 4. Indeed, in that portion of the board's opinion in which it reversed all of the examiner's rejections, the board held, on the record which contains appellants' declarations, that they, not Kaplan, invented the use of those mixed solvents, that appellants had antedated Kaplan as a reference under *35 USC § 102*(e), that the Kaplan patent cannot be used to [**22] show obviousness under § 103, and that appellants' claim was not obvious from a cited patent to Pruett et al. It also reversed a rejection under § § 102(g)/103 for obviousness which used Kaplan as the sole basis. Then it turned about and made an obviousness-type double patenting rejection based on Kaplan's claim 4. This rejection was predicated on the novel argument, particularly set out in the board's second opinion on rehearing, that Example 45 in Kaplan (which is *appellants' invention*, disclosed in Kaplan's patent to conform with the best mode requirement of § 112) "provides some of the support for the term 'organic solvent' as used in claim 4 of the Kaplan patent."

Thus, after concluding that the Kaplan patent is not available to show obviousness of appellants' claimed process, the board has nevertheless used Kaplan to show obviousness in a double patenting context, for it relied on no other reference. Moreover, that part of the Kaplan

disclosure used to do this is a description of appellants' joint invention. The board's claim-support theory does not suffice to justify this anomalous result. There is adequate support for the "organic solvent" limitation in claim 4 [**23] apart from appellants' specific *mixed* solvent invention, including the disclosure of the separate solvents in the mixture which are separately claimed by Kaplan. There is no way the board could have found appellants' claimed invention to be an obvious variation of what Kaplan claims except by treating the Kaplan patent disclosure as though it were prior art. This has repeatedly been held in our precedents to be impermissible. *In re Vogel; In re Aldrich; In re Boylan*, all supra. In effect, what the board did was to use a disclosure of appellants' own joint invention which had been incorporated in the Kaplan sole disclosure to show that their invention was but an obvious variation of Kaplan's claimed invention. That amounts to using an applicant's invention disclosure, which is not a 1-year time bar, as prior art against him. That is impermissible. D. *Chisum, Patents § 3.08*[2], § 5.03[3][f].

The PTO brief argues that *Vogel* sanctions such use of Kaplan's disclosure. We disagree. We do not find the factual situation here comparable to that in *Vogel*; neither was the reasoning of the board underlying the *Vogel* rejection comparable to the claim-supporting [**24] theory of the board in this case. Each double patenting rejection has to be decided on its own facts. *Vogel* dealt [*1581] with one difficult-to-analyze situation, this case presents a different one.

Summary

The double patenting rejection of appellants' single claim is *reversed* because the same invention is not being claimed, and because there is no proper evidence to show that the claim is for a mere obvious variation of what is claimed in the Kaplan patent relied on to support the rejection. There being no double patenting, the requirement for a terminal disclaimer was improper.

REVERSED

Crystallographic and Thermodynamic Comparison of Natural and Synthetic Ligands Bound to Streptavidin

P. C. Weber,* J. J. Wendoloski,† M. W. Pantoliano, and F. R. Salemme†

Contribution from The Du Pont Merck Pharmaceutical Company, P.O. Box 80228, Wilmington, Delaware 19880-0228. *Received July 11, 1991*

Abstract: The thermodynamic parameters of binding biotin and the dye 2-(4′-hydroxyphenylazo)benzoic acid (HABA) to streptavidin have been determined and are compared, together with X-ray crystal structures of the protein–ligand complexes and apostreptavidin. The X-ray crystal structures of apostreptavidin (space group *I*222, a = 94.2 Å, b = 104.0 Å, c = 47.7 Å, crystallographic R-factor = 0.209 at 1.84 Å resolution), a streptavidin–biotin complex (*I*222, a = 95.6 Å, b = 105.5 Å, c = 47.2 Å, R-factor = 0.171 at 1.55 Å resolution), and a streptavidin–HABA complex (*I*222, a = 95.1 Å, b = 105.6 Å, c = 47.4 Å, R-factor = 0.185 at 1.78 Å resolution) show common aspects of solvent displacement from the protein binding site together with preservation of an important interaction where the biotin ureido and HABA carboxylate oxygens bind in an oxyanion pocket formed by oriented hydrogen-bond donors in the binding site. Nevertheless, titrating calorimetric measurements show that biotin binding is enthalpically favored ($\Delta G°$ = −18.3 kcal/mol, $\Delta H°$ = −32.0 kcal/mol), while entropy terms dominate HABA binding ($\Delta G°$ = −5.27 kcal/mol, $\Delta H°$ = 1.70 kcal/mol).

Introduction

New pharmaceuticals are typically developed through screens that select active leads from a large compound library. In most cases, little is known about how interactions of a lead compound compare with those of the natural ligand bound to the target receptor. To investigate how dissimilar ligands are accommodated by a common binding site and better understand the principles of protein–ligand interactions applicable to rational drug design, we undertook a structural and thermodynamic comparison of biotin and an azobenzene dye bound to streptavidin.

Streptavidin is a tetrameric protein of M_r 64 000 that binds biotin with exceptionally high affinity ($K_a \simeq 10^{13}$ M^{-1}).[1] X-ray crystallographic studies of a streptavidin–biotin complex reveal a binding site that is complementary to biotin both in terms of specific hydrogen bonds made to biotin polar atoms and overall steric fit.[2] Organic dyes which differ structurally from biotin also bind to streptavidin. One of these, 2-(4′-hydroxyphenylazo)benzoic acid (HABA), has lower affinity than biotin (K_a = 10^4 M^{-1} (Table I)) but is quantitatively displaced when biotin binds, suggesting that both ligands share common binding sites.[1,3] Crystal structures of the streptavidin–biotin and streptavidin–HABA complexes reported here show that the ligands share an important interaction where the biotin ureido and HABA carboxylate oxygens bind in a common oxyanion pocket. Nevertheless, thermodynamic measurements show that the free energy of biotin binding is determined by enthalpy terms, while HABA binding is dominated by entropy.

Experimental Section

Crystallographic Studies. To facilitate structural comparisons between different streptavidin–ligand complexes, X-ray diffraction experiments were carried out with an orthorhombic crystal form,[4] previously used for an independent structure determination of the streptavidin–biotin complex,[5] that allows ligand diffusion into the crystal lattice. Orthorhombic crystals (space group *I*222, dimer per asymmetric unit) of apostreptavidin grown from ammonium sulfate solutions at 30 °C were soaked in saturating concentrations of biotin and HABA to prepare complex crystals.

X-ray data for apostreptavidin, biotin, and HABA complex crystals were collected using a Siemens imaging proportional counter and processed using XENGEN data reduction software.[6] Apostreptavidin unit cell parameters (a = 94.2, b = 104.0, c = 47.7 Å) changed slightly on soaking in ligand solutions (a = 95.1, b = 105.6, c = 47.4 Å, streptavidin–HABA complex; a = 95.6 Å, b = 105.5 Å, c = 47.2 Å, biotin complex). The apostreptavidin R_{sym}[7] value for 77 680 observations of 18 543 independent reflections (of 20 189 possible), where the average reflection intensity was greater than the background intensity, was 0.078 to 1.84 Å resolution. The streptavidin–biotin complex R_{sym} value for 129 058 observations of 32 621 independent reflections (35 118 possible) was 0.073 to 1.55 Å resolution, where the average intensity of reflections was 2.5 times the background intensity. The streptavidin–HABA complex R_{sym} value for 75 101 observations of 18 836 unique reflections was 0.092 to 1.84 Å resolution, where the average intensity of reflections was three times the background intensity.

Structures in the orthorhombic crystal form were determined by molecular replacement, using the biotin complex structure previously determined in space group $I4_122$ at 2.6 Å resolution as the probe molecule.[2] Identification of the noncrystallographic symmetry axis relating monomers in the orthorhombic crystal form defined symmetry-constrained rotational searches about the tetramer diad axes, together with translational searches along the unit cell a axis.[8] Initial searches in 3 degree and 1 Å increments produced a correlation coefficient of 0.56 using 4–5 Å resolution data, which increased to 0.79 when successively finer increments were searched. Eight cycles of restrained least-squares refinement[9] produced an initial model with a crystallographic R-factor[10] of 0.32 for 7036 reflections between 5.0 and 2.5 Å resolution, which was further reduced to R-factor = 0.29 using a molecular dynamics refinement protocol.[2]

The high resolution structures of apostreptavidin, and its complexes with biotin and HABA, were refined using restrained least-squares methods,[9] alternating with manual rebuilding cycles into $(F_o - F_c)\alpha_{calc}$ and $(2F_o - F_c)\alpha_{calc}$ electron density maps displayed with the graphics program FRODO.[11] Chemically identical monomers in the crystallographic asymmetric unit were refined independently, starting with ligand-free models. Ligand atoms and solvent molecules were introduced as they emerged as peaks greater than 3σ from the $(F_o - F_c)\alpha_{calc}$ electron density during successive refinement cycles. Refinement parameters for HABA were derived from the crystal structure of 4-(4′-hydroxy-

(7)

$$R_{sym} = \frac{\sum_{hkl}\sum_{i=1}^{N}|\langle I^{hkl}\rangle - \langle I_i^{hkl}\rangle|}{\sum_{hkl}\sum_{i=1}^{N} I_i^{hkl}}$$

* Author to whom correspondence should be addressed.
† Current address: Sterling Winthrop Research Division, 9 Great Valley Parkway, Malvern PA 19355.

(1) (a) Chaiet, L.; Wolf, F. J. *Arch. Biochem. Biophys.* 1964, *106*, 1-5. (b) Green, N. M. *Adv. Protein Chem.* **1975**, *29*, 85-133. (c) Green, N. M. *Methods Enzymol.* **1990**, *184*, 51-67.
(2) Weber, P. C.; Ohlendorf, D. H.; Wendoloski, J. J.; Salemme, F. R. *Science* **1989**, *243*, 85-88.
(3) Green, N. M. *Biochem. J.* **1965**, *94*, 23c-24c.
(4) Pahler, A.; Hendrickson, W. A.; Kolks, M. A. G.; Argarana, C. E.; Cantor, C. R. *J. Biol. Chem.* **1987**, *262*, 13933-13937.
(5) Hendrickson, W. A.; Pahler, A.; Smith, J. L.; Satow, Y.; Merritt, E. A.; Phizackerley, R. P. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 2190-2194.
(6) Howard, A. J.; Gilliland, G. L.; Finzel, B. C.; Poulos, T. L.; Ohlendorf, D. H.; Salemme, F. R. *J. Appl. Crystallogr.* **1987**, *20*, 383-387.
(8) Fujinaga, M.; Read, R. *J. Appl. Crystallogr.* **1987**, *20*, 517-521.
(9) Hendrickson, W. A.; Konnert, J. H. *Biomolecular Structure, Function, Conformation and Evolution*; R. Srinivasan: Oxford, 1980; pp 43-57.
(10) Crystallographic R-factor = $\sum|F_o - F_c|/\sum F_o$.
(11) Jones, T. A. *J. Appl. Crystallogr.* **1978**, *11*, 268-272.

0002-7863/92/1514-3197$03.00/0

Table I. Thermodynamic Parameters for Streptavidin–Ligand Interactions

ligand	K_a[a] (M^{-1})	n	$\Delta G°$ (kcal/mol)	$\Delta H°$ (kcal/mol)	$T\Delta S°$ (kcal/mol)	χ^2
HABA	7295 (±554)	1.12 (±.07)	−5.27 (±.05)	1.70 (±.01)	6.97 (±.04)	0.080
HABA[b]	10000		−5.46			
d-biotin[c]	2.5×10^{13}	0.86	−18.3	−32.0	−13.7	

[a] Data for average of three experiments (error parameters of ±1 standard deviation are in parentheses). χ^2 gives the average deviation of data points for computed best fit for 18 injection aliquots in each experiment, assuming independent binding sites on the streptavidin tetramer. [b] Literature values for HABA.[1c] [c] Only $\Delta H°$ and n were fitting parameters for the observed reaction heat for d-biotin with K_a fixed at the literature value of 2.5×10^{13} (from ref 1c).

phenylazo)benzoic acid.[12] Aromatic ring geometry was constrained during the protein complex refinement. Planarity constraints were omitted from the azo group linking the HABA aromatic rings.

The apostreptavidin structure (crystallographic R-factor = 0.209 at 1.84 Å resolution) incorporates 256 water molecules, residues 13–65, 68–133 in molecule 1 and residues 13–46, 52–65, 69–133 in molecule 2 in the crystallographic asymmetric unit (residues 13–138 constitute "core" streptavidin found in commercial preparations[4]). The streptavidin–biotin complex structure (R-factor = 0.171 at 1.55 Å resolution) incorporates all protein atoms corresponding to residues 13–133 of the mature streptavidin sequence and 209 water molecules. The streptavidin–HABA complex structure was refined to a final crystallographic R-factor of 0.185 at 1.78 Å resolution. Several residues located at the amino termini or in surface loops (residues 13, 14, 49, 66, 67 in molecule 1 and residues 13, 14, 66–68 in molecule 2 of the asymmetric unit) are poorly defined in the streptavidin–HABA complex and are not included in the model. This model also includes 209 water molecules. Coordinates have been deposited in the Brookhaven Protein Data Bank.[13]

Calorimetric Studies. Streptavidin from *Streptomyces avidinii* was purchased from Calbiochem (La Jolla, CA) and used without further purification. HABA and d-biotin were purchased from Fluka (Ronkonkoma, NY) and Sigma (St. Louis, MO), respectively, and used without further purification.

Streptavidin solutions were titrated by addition of 18×15 μL aliquots of ligand solution at 5-min intervals at 25 °C in a MicroCal (Northampton, MA) Omega titration calorimeter. K_a, $\Delta H°$, and n (stoichiometry per subunit) were obtained through nonlinear least-squares fit of the observed reaction heat for each titration step.[14] Lyophilized streptavidin was dissolved in unbuffered 100 mM KCl at a concentration of between 4 and 188 mM (Given four biotin binding sites in the streptavidin tetramer, the concentration of ligand binding sites ranged from 15 to 750 mM.) and dialyzed overnight against this same solution. All calorimetry experiments were conducted in 100 mM KCl in the absence of buffer to avoid heat effects due to ionization of buffer components. The pH was adjusted using small amounts of 1 M NaOH and 1 M HCl, and the protein was equilibrated with this solution by dialysis. The pH 6.9 was chosen to insure the full protonation of the phenolic hydroxyl of HABA, as expected from the reported pK_a of 8.2[15] to 8.5,[16] so that heat effects due to ionization of the ligand are negligible. Furthermore, control experiments where HABA was diluted into 0.1 M KCl and 0.1 M KCl into protein were performed to correct for the small contributions of heat of dilution. Protein concentrations were determined by amino acid analysis, while ligand concentrations were gravimetrically measured. The solid ligands were dissolved in the dialyzate that resulted from the streptavidin dialysis to insure composition identity with respect to all solution components other than the reactants.

For the experiments with HABA it was necessary to use relatively high concentrations of streptavidin (150–188 mM) because of the weak binding and small experimental signal (heat of reaction). The HABA concentration was 10 times that of streptavidin to insure that the observed heats of reaction would be 120–150 μcal/injection at the beginning of the titration and that about 80% of the ligand added per injection was bound by the protein for the first few injections. Moreover, these conditions also insured that about 80% of the binding sites would be saturated after the last injection. The control titration of HABA into 100 mM KCl yielded only ~3 μcal/injection for all 18 injections and indicate that the observed heats of reaction for HABA binding to streptavidin are well above the background signal.

Results and Discussion

Ligand binding affinity represents the difference in free energy between the protein plus unbound ligand in solution and their complex. As inferred from previous studies at 2.6 Å resolution[2] and verified at high resolution (Figure 1a), streptavidin provides a binding site for biotin whose interactions stabilize a resonance form of biotin which makes better hydrogen bonds with the protein than are possible with biotin in water. This is achieved through polarization of the biotin ureido group so that negative charge is localized on the ureido oxygen (Figure 2). The polarizability of ureas is known from small molecule X-ray studies showing that bond lengths and corresponding atomic charge densities vary in response to the strength of the hydrogen bonding partners.[17] Although expected changes in bond lengths are too small to be reliably determined from the streptavidin–biotin crystal structure, localization of negative charge on the biotin ureido oxygen is reflected by its tetrahedral (sp^3) coordination to three protein side chains that donate hydrogen bonds and form an oxyanion pocket for the ureido oxygen (Figure 3). As reported earlier, all of the groups that hydrogen bond to biotin are additionally hydrogen-bonded to other protein groups, forming a rigid lattice that is also present in apostreptavidin.[18]

Additional factors contributing to energetics of ligand binding include favorable entropy effects arising from release of five water molecules ordered in the biotin site of apostreptavidin (including one water with unit occupancy and low B-value located in the oxyanion pocket) and burying hydrophobic surface area of biotin that was accessible in solution. Opposing entropy effects could arise from biotin immobilization and ordering of loop residues 46–50 on biotin binding.[2] However, the measured free energy of biotin binding to streptavidin ($\Delta G° = -18.3$ kcal/mol) is almost wholly enthalpic, $\Delta H° = -32.0$ kcal/mol (Table I), indicating that net entropy contributions to biotin binding are unfavorable. Taken together, these results suggest that the dominant effect contributing to biotin binding is enhanced hydrogen bonding made between the ligand and protein, due particularly to stabilization of a biotin resonance form whose tetrahedrally coordinated, sp^3 ureido oxygen makes more and stronger hydrogen bonds than an sp^2 oxygen forms with water in solution.

HABA binds streptavidin with minimal alteration of the binding site geometry (Figure 3). This is probably to be expected, given the extensive pattern of binding site interactions outlined previously.[2] HABA is oriented in the binding site so that the position of one of its benzoate oxygen atoms coincides with that of the biotin ureido oxygen, while the remainder of the linked ring system roughly approximates the orientation of the fused biotin rings and aliphatic valaryl side chain (Figures 1 and 3). HABA binding does not completely immobilize the loop comprising residues 46–50 which is disordered in apostreptavidin but becomes immobilized on binding biotin.[2] This loop remains partially disordered in the HABA–streptavidin complex and allows some solvent

(12) Harlow, R. L.; Simons, D. M.; Weber, P. C. *Acta Crystallogr.* **1992**, *C48*, 48–50.

(13) Bernstein, F. C.; Koetzle, T. F.; Williams, G. J. B.; Meyer, E. F., Jr.; Brice, M. D.; Rogers, J. R.; Kennard, O.; Shimanouchi, T.; Tasumi, M. *J. Mol. Biol.* **1977**, *112*, 535–542.

(14) (a) Wiseman, T.; Williston, S.; Brandts, J. F.; Lin, L. *Anal. Biochem.* **1989**, *179*, 131–137. (b) Brandts, J. F.; Lin, L.-N.; Wiseman, T.; Williston, S.; Yang, C. P. *Am. Laboratory* **1990**, *22*, 30–41. (c) Connelly, P. R.; Varadarajan, R.; Sturtevant, J. M.; Richards, F. M. *Biochemistry* **1990**, *29*, 6108–6114.

(15) (a) Baxter, J. H. *Arch. Biochim. Biophys.* **1964**, *108*, 375–383. (b) Thomas, E. W.; Merlin, J. C. *Spectrochim. Acta* **1979**, *35A*, 1251–1255. (c) Ni, F.; Cotton, T. M. *J. Raman Spectrosc.* **1988**, *19*, 429–438.

(16) Green, N. M. *Methods Enzymol.* **1970**, *18A*, 418–424.

(17) Blessing, R. H. *J. Am. Chem. Soc.* **1983**, *105*, 2776–2783.

(18) The oxyanion pocket is analogous to the oxyanion hole in serine proteases (Kraut, J. *Annu. Rev. Biochem.* **1977**, *46*, 331–358) that stabilizes the tetrahedral acyl enzyme intermediate. Both sites incorporate backbone NH groups to indirectly or directly orient hydrogen bond donors toward the oxyanion.

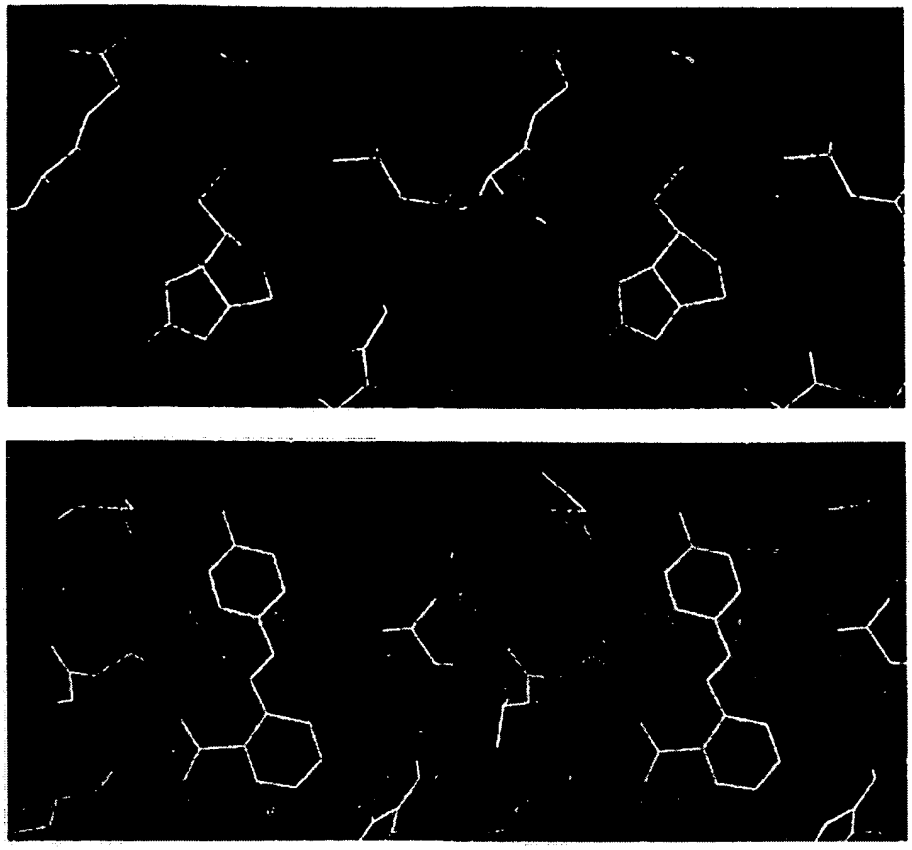


Figure 1. Stereoscopic views of $(2F_o - F_c)\alpha_{calc}$ electron density for ligands binding to streptavidin: (a, top) biotin at 1.55 Å resolution and (b, bottom) the dye 2-(4′-hydroxyphenylazo)benzoic acid (HABA) at 1.78 Å resolution. Spectroscopic studies indicate that the dye binds as the hydrazone, as also suggested by the tetrahedral character of the azo nitrogen nearest the benzoic acid ring. Less defined electron density for the hydroxyphenyl ring of the dye suggests that it is less constrained than the benzoic acid ring when bound to the protein.

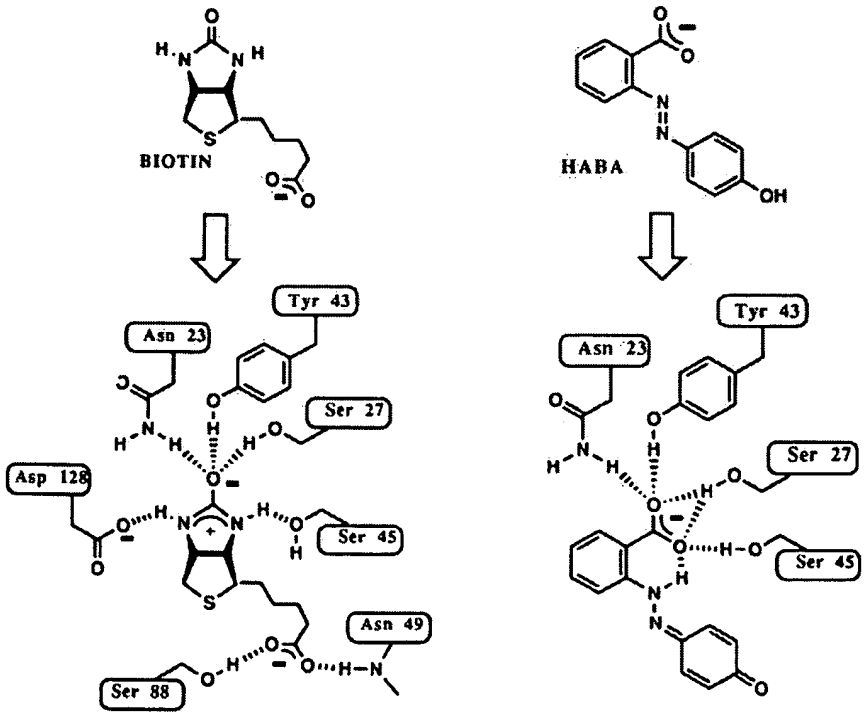


Figure 2. Schematic illustrations of biotin (left) and HABA (right) binding to streptavidin. Biotin is bound as a minor resonance form owing to specific protein interactions that polarize the ureido group and allow strong hydrogen bond formation with residues (Asn23, Ser27, Tyr43) in the oxyanion pocket. HABA binds as a hydrazone tautomer, in part because the hydrazone NH proton can stabilize the charge or orientation of the carboxyl group in the hdyrophobic environment of the binding site.

exposure of the HABA hydroxyphenyl group, although the same five water molecules that are displaced by biotin are also displaced on HABA binding.

Spectroscopic studies indicate that HABA binds as the hydrazone tautomer, which is favored in low dielectric environments because the hydrazone NH can form an internal hydrogen bond

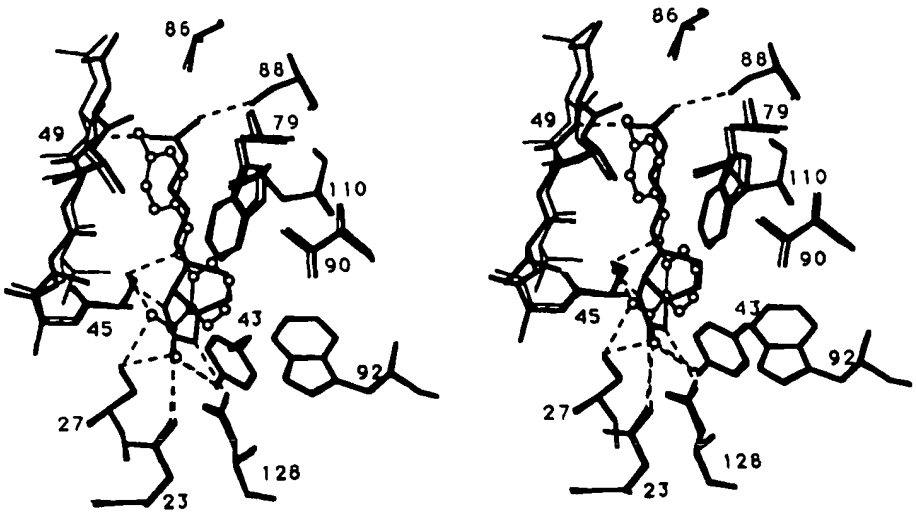


Figure 3. Stereoscopic view showing a superposition of the streptavidin–biotin (wider lines) and streptavidin–HABA (thinner lines) complexes. Open circles indicate positions of HABA atoms. Most features of the binding site are conserved despite differences in ligand structure. Minor differences include a small displacement of residues 47–51 in the streptavidin–HABA complex to accommodate the hydroxyphenyl ring of HABA. Thin dashed lines show hydrogen bonds between ligand and protein.

that helps stabilize the benzoate negative charge (Figure 2).[15] Hydrazone binding is consistent with the refined electron density for the ligand (Figure 1b), which shows tetrahedral character at the nitrogen nearest the benzoic acid ring. One benzoate oxygen accepts hydrogen bonds from Asn23, Tyr43, and Ser27 in the oxyanion pocket, while the other shares a hydrogen bond from Ser27 and accepts a hydrogen bond from the hydroxyl of Ser45, together with the internal hydrogen bond from the hydrazone NH (Figure 2). There is no analogue of the biotin–Asp128 interaction in HABA binding, and it is notable that Ser45 reorients so that it donates, rather than accepts, a hydrogen bond to the ligand (Figures 2 and 3). Despite these differences, it is somewhat surprising that HABA binding to streptavidin (ΔG° = −5.27 kcal/mol (Table I)) is dominated by the entropy terms ($T\Delta S^\circ$ = 6.97 at 25 °C (Table I)). This suggests that despite the preservation of key hydrogen-bond interactions with residues in the oxyanion pocket, hydrophobic interactions predominate in the energetics of HABA binding.

One source of the difference in binding energetics is the extent of ligand polarization possible in biotin versus HABA. Computational estimates of binding energy show substantial increases for biotin resonance forms that localize negative charge on the ureido oxygen and positive charge on the ureido nitrogen atoms,[19] an anticipated result since all of these atoms interact with complementary hydrogen-bond donors or acceptors in the binding site (Figure 2). These interactions underlie streptavidin's ability to stabilize a biotin resonance form that makes more and stronger hydrogen bonds with the protein than the ligand makes with solvent. Although HABA makes geometrically similar interactions with streptavidin, in contrast to biotin there are no resonant charge distributions for the tautomer that produce comparable enhancements in the number or strength of hydrogen bonds that HABA makes to the protein versus solvent. Redistributions of charge in the benzoate carboxyl group that potentially enhance interactions with residues in the oxyanion pocket, for example, attenuate interactions with Ser45, and vice versa. Consequently, while the geometry of the oxyanion binding interactions looks similar for biotin and HABA, their energetics are quite different. Both HABA hydrazone formation and oxyanion pocket interactions reflect a compromise between solution and bound conformations that have little net stabilizing effect on complex formation,[20] while binding is determined by water displacement, hydrophobic forces, and interactions like stacking of the HABA hydroxyphenyl ring with the indole ring of Trp79 (Figures 1b and 3). Most interesting from the drug design standpoint are the fundamental differences in energetics and driving forces for the natural and "screened" ligand. Although this is an implicit assumption of many mechanism-based or structural approaches to ligand design, it seems clear that very different energetics frequently accompany structurally similar protein–ligand interactions.[20] At the same time, plausible strategies for increasing affinity of the HABA "lead compound" may be successful, although they exploit the unique properties of the streptavidin binding site in ways that are different, if not as ultimately effective as the natural ligand.

(19) Point charge distributions for biotin, HABA and HABA hydrazone were computed using a 4-31 basis set and the program GAMESS (Dupius, M.; Spangler, D.; Wendoloski, J. J. NRCC Program QC01). Estimates of ligand–protein interaction energies were made from complex crystal coordinates using the AMBER potential [Weiner, P.; Kollman, P. A. *J. Comput. Chem.* **1981**, *2*, 287–303. Weiner, S. J.; Kollman, P. A.; Case, D. A.; Singh, U. C.; Ghio, C.; Algona, G.; Weiner, P. *J. Am. Chem. Soc.* **1984**, *106*, 765–784.] for the protein and computed parameters for the ligands. Effects of ligand charge redistribution were estimated by scaling charges on the biotin ureido oxygen–carbon and nitrogen–hydrogen atom pairs to retain local and overall ureido group neutrality. Discounting charge reorganization in the protein, which could be significant, the biotin–streptavidin electrostatic interaction increased 24 kcal/mol per unit excess negative charge localized on the ureido oxygen.

(20) Morgan, B. P.; Scholtz, J. M.; Ballinger, M. D.; Zipkin, I. D.; Bartlett, P. A. *J. Am. Chem. Soc.* **1991**, *113*, 297–306.

J|A|C|S
ARTICLES
Published on Web 01/27/2005

Solutes Probe Hydration in Specific Association of Cyclodextrin and Adamantane

Daniel Harries,* Donald C. Rau, and V. Adrian Parsegian

Contribution from the Laboratory of Physical and Structural Biology, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892-0924

Received July 24, 2004; E-mail: harries@helix.nih.gov

Abstract: Using microcalorimetry, we follow changes in the association free energy of β-cyclodextrin (CD) with the hydrophobic part of adamantane carboxylate (AD) due to added salt or polar (net-neutral) solutes that are excluded from the molecular interacting surfaces. Changes in binding constants with solution osmotic pressure (water activity) translate into changes in the preferential hydration upon complex formation. We find that these changes correspond to a release of 15−25 solute-excluding waters upon CD/AD association. Reflecting the preferential interaction of solute with reactants versus products, we find that changes in hydration depend on the type of solute used. All solutes used here result in a large change in the enthalpy of the CD−AD binding reaction. In one class of solutes, the corresponding entropy change is much smaller, while in the other class, the entropy change almost fully compensates the solute-specific enthalpy. For many of the solutes, the number of waters released correlates well with their effect on air−water surface tensions. We corroborate these results using vapor pressure osmometry to probe individually the hydration of reactants and products of association, and we discuss the possible interactions and forces between cosolute and hydrophobic surfaces responsible for different kinds of solute exclusion.

Introduction

Celebrated for their unique ability to enhance the solubility of nonpolar "hydrophobic" organic solutes, naturally produced cyclodextrins (CDs) find use in such diverse fields as pharmaceutical, cosmetic, and food industries.[1−8] Shaped as a hollow truncated cone, this cyclic carbohydrate is unique in that it can incorporate nonpolar "guest" molecules in its central cavity to form noncovalent "guest−host" inclusion complexes. Because CD is quite soluble in water, the inclusion complex confers this property on the less soluble guest. Depending on both the CD derivative and host molecule, the association constants for the inclusion complex are typically 10^3-10^5 M^{-1}. Three types of cyclodextrin are naturally most abundant: α-, β-, and γ-CD, containing 6, 7, and 8 α-D-glucose units, respectively. In addition to these naturally occurring species, many synthetic modifications of CD have been produced to increase water solubility and association specificity.

Because CD specifically incorporates nonpolar hydrophobic solutes, it is also attractive as a model system for hydrophobic interactions and their role in determining specificity in biologically relevant systems, such as specific protein−ligand interactions.[9−12] Guest−host association must be accompanied by a release of surface/cavity neighboring waters. Crystal structures show that at full hydration, the cavity of β-CD (on which we focus here) accommodates $\approx$11 water molecules;[13,14] a similar number can be expected to be released upon association as the guest displaces some or all of these waters.[15] Moreover, a large interacting guest must also shed some or all of its surface waters in order to complex.

More evidence for the anticipated water release comes from the heats of complexation. These measurements show that the CD's interaction with guest molecules has a negative heat capacity, often related to burial of nonpolar surfaces.[16−19] Furthermore, with different solvent conditions and guest molecules, changes in reaction free energy often show "entropy−enthalpy compensation".[20−26] The large residual entropic term,

(1) Rekharsky, M. V.; Inoue, Y. *Chem. Rev.* **1998**, *98*, 1875−1917.
(2) Wenz, G. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 803−822.
(3) Albers, E.; Muller, B. W. *Crit. Rev. Ther. Drug Carrier Syst.* **1995**, *12*, 311−337.
(4) Saenger, W. *Angew. Chem., Int. Ed. Engl.* **1980**, *19*, 344−362.
(5) Becheri, A.; Nostro, P. L.; Ninham, B. W.; Baglioni, P. *J. Phys. Chem. B* **2003**, *107*, 3979−3987.
(6) Banerjee, I. A.; Yu, L.; Matsui, H. *J. Am. Chem. Soc.* **2003**, *125*, 9542−9543.
(7) Schaschke, N.; Fiori, S.; Weyher, E.; Escrieut, C.; Fourmy, D.; Muller, G.; Moroder, L. *J. Am. Chem. Soc.* **1998**, *120*, 7030−7038.
(8) Brewster, M. E.; Loftsson, T. *Pharmazie* **2002**, *57*, 94−101.
(9) Iglesias, E. *J. Am. Chem. Soc.* **1998**, *120*, 13057−13069.
(10) Kushiro, T.; Nambara, E.; McCourt, P. *Nature* **2003**, *422*. 122.
(11) Ross, P. D.; Rekharsky, M. V. *Biophys. J.* **1996**, *71*, 2144−2154.
(12) Auletta, T.; de Jong, M.; Mulder, A.; van Veggel, F.; Huskens, J.; Reinhoudt, D.; Zou, S.; Zapotoczny, S.; Schonherr, H.; Vancso, G.; Kuipers, L. *J. Am. Chem. Soc.* **2004**, *126*, 1577−1584.
(13) Steiner, T.; Mason, S. A.; Saenger, W. *J. Am. Chem. Soc.* **1991**, *113*, 5676−5687.
(14) Steiner, T.; Saenger, W. *Mol. Phys.* **1991**, *72*, 1211−1232.
(15) Saudan, C.; Dunand, F.; Abou-Hamdan, A.; Bugnon, P.; Lye, P.; Lincoln, S.; Merbach, A. *J. Am. Chem. Soc.* **2001**, *123*, 10290−10298.
(16) Spolar, R. S.; Livingstone, J. R.; Record, M. T. *Biochemistry* **1992**, *31*, 3947−3955.
(17) Myers, J. K.; Pace, C. N.; Scholtz, J. M. *Protein Sci.* **1995**, *4*, 2138−2148.
(18) Murphy, K. P.; Freire, E. *Adv. Protein Chem.* **1992**, *43*, 313−361.
(19) Makhatadze, G. I.; Privalov, P. L. *Adv. Protein Chem.* **1995**, *47*, 307−425.
(20) Harrison, J. C.; Eftnik, M. R. *Biopolymers* **1982**, *21*, 1153−1166.
(21) Eftink, M.; Andy, M.; Bystrom, K.; Perlmutter, H.; Kristol, D. *J. Am. Chem. Soc.* **1989**, *111*, 6765−6772.
(22) Inoue, Y.; Hakushi, T.; Liu, Y.; Tong, L. H.; Shen, B. J.; Jin, D. S. *J. Am. Chem. Soc.* **1993**, *115*, 475−481.

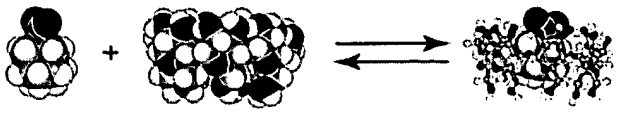


Figure 1. Schematic of the complexation reaction between adamantane carboxylate and β-cyclodextrin. Drawn to scale, the figure shows the close fit of AD in the CD cavity, requiring the release of surface-hydrating waters from both interacting molecular surfaces.

expected (through extrapolation) to persist even in the (hypothetical) absence of heat release, was proposed to indicate water release upon association.[22]

We study the complexation of CD with adamantane carboxylate (AD) acting as a guest. Due to its charged group, AD is water soluble, despite its substantial globular hydrophobic region. The β-CD•AD inclusion complex, shown schematically in Figure 1, is highly favorable, with an association constant of $K_a \approx 5 \times 10^4\ M^{-1}$ at room temperature. The size of AD is almost exactly accommodated by the cavity of β-CD. NMR experiments show that once complexed, AD fits deep in the CD cavity, with the charged carboxylate group exposed to solution at the wider opening of the host CD.[27]

There are many definitions for waters of "hydration" depending on the probing technique and the energetic or structural criteria applied.[28-35] We use the response to osmotic stress as an operational definition of hydration.[36-39] Many salts and polar solutes are preferentially excluded from hydrophobic surfaces.[40-43] The solubility of many hydrophobic compounds, for example, decreases with increasing concentration of salts or polar solutes due to this unfavorable interaction.[44] A common feature of this exclusion is that the apparent number of solute excluding waters does not vary much with salt or polar solute concentration. The number of these preferentially bound waters, however, sensitively depends on the chemical nature and size of the probing solute as well as on the nature of the macromolecular surface.[37,41-43,45-48] Changes in the number of the preferentially bound waters accompanying binding reactions can be measured from the change in binding free energies ($\Delta G°$) with solute concentration.[37,40,49-51] For a constant difference in the number of included waters, $\Delta G°$ will vary linearly with solute osmotic pressure.

To probe the changes in preferential hydration involved in the CD/AD association, we use two different experimental approaches. The two approaches give a complementary and consistent picture of water release upon complex formation.

In the first approach, we use microcalorimetry to determine equilibrium constants for complexation in the presence of an additional solute (cosolute). We find that for a wide range of cosolutes, the association free energy varies linearly with water chemical potential (or osmotic pressure). This translates into a constant change in the number of cosolute-excluding waters in complexation. However, we find that this difference in numbers of released waters depends on the type of cosolute probing the reaction, reflecting the preferential interaction of cosolutes with (or extent of exclusion from) the complexing molecules.

Using calorimetry, we can also follow individually changes in heats ($\Delta H°$) and entropies ($T\Delta S°$) of association. We find that all cosolutes we have used belong to one of two classes. The first class has a strong enthalpic and smaller entropic contribution to the binding free energy. The second class has a strong enthalpic contribution to the free energy that is almost completely compensated by the entropic change. The dissection of free energy changes into the enthalpic and entropic contributions enables us to discuss the possible intermolecular forces responsible for the different preferential interactions between cosolutes and CD/AD.

In the second experimental approach, we determine the preferential hydration of the individual molecular species using vapor pressure osmometry of CD and AD solutions with added cosolutes. The number of solute-excluding waters associated with each species can be determined from changes in solution osmolalities (osmotic pressures) of mixed solute−cosolute solutions as developed by Courtenay et al.[41,52] Evaluated differences between cosolute-excluding waters of reactants (CD or AD) and products (CD•AD complex) are then simply translated into the changes in hydration upon complexation.

Results and Analysis

Changes in Molecular Association from Calorimetry. Using isothermal titration calorimetry (ITC), we follow the change in heat release, Q, with each injection of CD into a solution of AD. Figure 2 shows three typical plots of the heat release per mole of CD solution, integrated for each injection. The experiment is repeated in the presence of cosolute (glycine in Figure 2) in both CD (injectant) and AD (cell) solutions at the same molal concentrations. One prominent effect of glycine addition is to increase the amount of heat released in the CD/AD association, as seen in the value of heat release for the first injections in Figure 2.

(23) Rekharsky, M. V.; Inoue, Y. *J. Am. Chem. Soc.* **2002**, *124*, 12361−12371.
(24) Rekharsky, M.; Inoue, Y. *J. Am. Chem. Soc.* **2000**, *122*, 4418−4435.
(25) Godinez, L. A.; Schwartz, L.; Criss, C. M.; Kaifer, A. E. *J. Phys. Chem. B* **1997**, *101*, 3376−3380.
(26) Gelb, R. I.; Alper, J. S. *J. Phys. Org. Chem.* **1995**, *8*, 825−832.
(27) Rudiger, V.; Eliseev, A.; Simova, S.; Schneider, H. J.; Blandamer, M. J.; Cullis, P. M.; Meyer, A. J. *J. Chem. Soc., Perkin Trans.* **1996**, *2*, 2119−2123.
(28) Clarke, C.; Woods, R. J.; Gluska, J.; Cooper, A.; Nutley, M. A.; Boons, G. J. *J. Am. Chem. Soc.* **2001**, *123*, 12238−12247.
(29) Chalikian, T. V. *J. Phys. Chem. B* **2001**, *105*, 12566−12578.
(30) Chatake, T.; Ostermann, A.; Kurihara, K.; Parak, F. G.; Niimura, N. *Proteins* **2003**, *50*, 516−523.
(31) Tarek, M.; Tobias, D. J. *Biophys. J.* **2000**, *79*, 3244−3257.
(32) Biswal, B. K.; Sukumar, N.; Vijayan, M. *Acta Crystallogr., Sect. D* **2000**, *56*, 1110−1119.
(33) Liu, D. F.; Wyttenbach, T.; Barran, P. E.; Bowers, M. T. *J. Am. Chem. Soc.* **2003**, *125*, 8458−8464.
(34) Sanjeev, B. S.; Vishveshwara, S. *Proteins* **2004**, *55*, 915−923.
(35) Southall, N. T.; Dill, K. A.; Haymet, A. D. J. *J. Phys. Chem. B* **2002**, *106*, 521−533.
(36) Parsegian, V. A.; Rand, R. P.; Rau, D. C. Macromolecules and water probing with osmotic pressure. In *Methods in Enzymology*; Johnson, M., Ackers, G., Eds.; Academic Press: San Diego, CA, 1995; Vol. 257.
(37) Parsegian, V. A.; Rand, R. P.; Rau, D. C. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 3987−3992.
(38) Sidorova, N. Y.; Rau, D. C. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 12272−12277.
(39) Parsegian, V. A. *Int. Rev. Cytol.* **2002**, *215*, 1−31.
(40) Timasheff, S. N. *Adv. Protein Chem.* **1998**, *51*, 355−432.
(41) Courtenay, E. S.; Capp, M. W.; Anderson, C. F.; Record, M. T. *Biochemistry* **2000**, *39*, 4455−4471.
(42) Qu, Y.; Bolen, C. L.; Bolen, D. W. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, 9268−9273.
(43) Baldwin, R. L. *Biophys. J.* **1996**, *71*, 2056−2063.
(44) Collins, K.; Washabaugh, M. *Q. Rev. Biophys.* **1985**, *18*, 323−422.
(45) Timasheff, S. N. *Adv. Protein Chem.* **1998**, *51*, 355−432.
(46) Bolen, D. W.; Baskakov, I. V. *J. Mol. Biol.* **2001**, *310*, 955−963.
(47) Ellis, R. J.; Minton, A. P. *Nature* **2003**, *425*, 27−28.
(48) Bostrom, M.; Williams, D. R. M.; Ninham, B. W. *Biophys. J.* **2003**, *85*, 686−694.
(49) Garner, M. M.; Rau, D. C. *EMBO J.* **1995**, *14*, 1257−1263.
(50) Reid, C.; Rand, R. P. *Biophys. J.* **1997**, *72*, 1022−1030.
(51) Fini, P.; Castagnolo, M.; Catucci, L.; Cosma, P.; Agostiano, A. *J. Therm. Anal.* **2003**, *73*, 653−659.
(52) Courtenay, E. S.; Capp, M. W.; Record, M. T. *Protein Sci.* **2001**, *10*, 2485−2497.

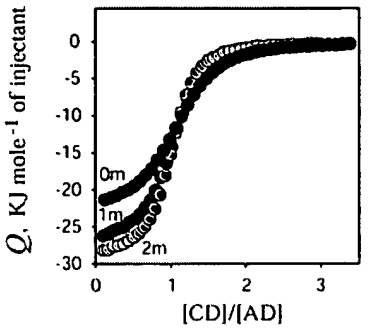


Figure 2. Effect of glycine (cosolute) concentration on heat release in CD/AD complexation measured using ITC. The heat release for each injection of CD solution into AD is shown for three molal concentrations of glycine: 0 m (red), 1 m (blue), and 2 m (green). All measurements were made at 30 °C.

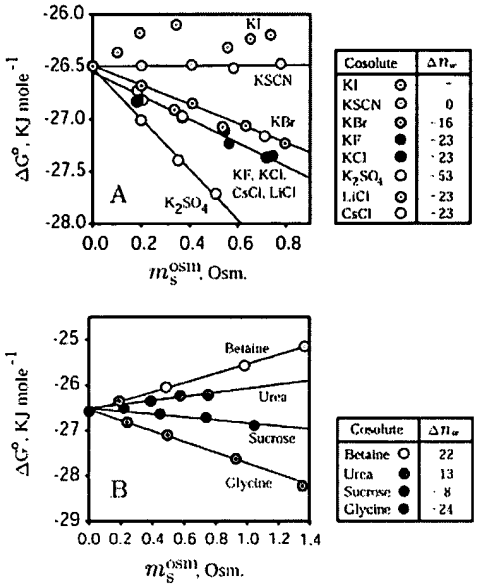


Figure 3. Changes in the number of cosolute-excluding waters (Δn_w) in CD/AD association evaluated from changes in association free energies ($\Delta G°$) with changes in cosolute osmolal concentration (m_s^{osm}) for β-CD (eq 1) with different (A) salts and (B) neutral cosolutes. All measurements were made at 30 °C.

The plots in Figure 2 can be well fit by a 1:1 CD/AD binding model, from which we determine the free energy ($\Delta G°$) of complex formation, AD + β-CD ⇌ β-CD•AD. Figure 3 shows $\Delta G°$ versus the solute osmotic pressure expressed in terms of osmolal concentration for several different cosolutes. Free energies are shown for both (charged) salts (Figure 3A) and net-neutral (Figure 3B) cosolutes. For all cosolutes, except KI, we find a linear dependence of the binding free energy with cosolute concentration.

The action of cosolutes on the reaction can be analyzed in terms of either a difference in the number of associated cosolutes between products and reactants, Δn_s, or a difference in the number of associated waters, Δn_w. The two approaches are necessarily connected through the Gibbs–Duhem equation, relating the concurrent changes in μ_w and μ_s, water and cosolute chemical potential, respectively, due to cosolute addition.

The number of cosolute-excluding waters, Δn_w, released in complexation is related to the change in binding free energy with changes in water chemical potential, μ_w, by[36,37,41,49–51]

$$\Delta n_w = -\frac{d\Delta G°}{d\mu_w} = \frac{55.6}{RT}\frac{d\Delta G°}{dm_s^{osm}} \quad (1)$$

Here T is the absolute temperature, and R the ideal gas constant; 55.6 is the number of moles of water in 1 Kg, and m_s^{osm} is the solute osmolal concentration, a measure of water chemical potential. If CD/AD concentrations are much smaller than cosolute concentration, then $dm_s^{osm} = -(55.6/RT)d\mu_w$.

In the limit of low CD/AD concentrations, Δn_w is also related to Δn_s, the change in the excess number of cosolutes associated with, or preferentially excluded from, CD/AD.[39–41] Again, the link between Δn_s and Δn_w is made using the Gibbs–Duhem relationship in this limit of low CD/AD concentration, $m_w d\mu_w + m_s d\mu_s = 0$, where m_w and m_s are water and solute molal concentrations, respectively. Therefore, we find

$$\Delta n_s = -\frac{d\Delta G°}{d\mu_s} = \Delta n_w \frac{d\mu_w}{d\mu_s} \cong -\frac{m_s \Delta n_w}{55.6} \quad (2)$$

Note that in this limit, Δn_s is also directly related to the difference in Γ_s, the preferential interaction coefficient, between products and reactants, $\Delta\Gamma_s = \Delta n_s$.[39–42,53]

The linearity of the plots shown in Figure 3, even in the limit $m_s \to 0$, translates using eq 1 into a constant change in the number of cosolute-excluding waters upon binding Δn_w. Conversely, using eq 2, we find that the change in associated cosolute, Δn_s, between reactants and products must vary linearly with cosolute osmolality. We focus here on Δn_w because it remains constant as cosolute concentration is varied. The reactants and products are not single species, but rather a distribution of conformations and geometries, as observed for products by NMR.[27] The measured Δn_w may include changes in the probabilities of these configurations. From the thermodynamic analysis, however, we find that the change in hydration upon association is constant, even at the highest cosolute concentrations we have used, as seen in the linearity of the sets in Figure 3. This indicates that if, indeed, different complex geometries are preferred at different cosolute concentrations, all shed the same number of waters upon complexation.

For salts (Figure 3A), we find that the number of excluded waters depends on the ionic species, reflecting different extents of exclusion from CD/AD interacting surfaces. The extent of exclusion depends more sensitively on the anion than the cation and generally follows the classical Hofmeister series ordering.[44,54,55] For LiCl, KCl, and CsCl, we find that $\Delta n_w \approx -23$ waters are released in complexation; for K_2SO_4, we find $\Delta n_w = -53$ water molecules displaced; for KF and KCl, $\Delta n_w = -23$, and for KBr, $\Delta n_w = -16$. In solutions of KSCN, no change is observed in the association constant, corresponding to $\Delta n_w = 0$. Finally, in KI, a weak nonlinear relationship is found, possibly reflecting association of I_n^- clusters or molecular I_2 with the CD, and subsequent competition with AD for binding.

For the neutral cosolutes shown in Figure 3B, there is also a wide range of exclusion or inclusion reflected in the changes in numbers of cosolute-excluding waters upon complexation.

(53) Kita, Y.; Arakawa, T.; Lin, T.-Y.; Timasheff, S. N. *Biochemistry* **1994**, *33*, 15178–15189.
(54) Hofmeister, F. *Arch. Exp. Pathol. Pharmakol.* **1887**, *24*, 247–260.
(55) Kunz, W.; Lo Nostro, P.; Ninham, B. *Curr. Opin. Colloid Interface Sci.* **2004**, *9*, 1–18.

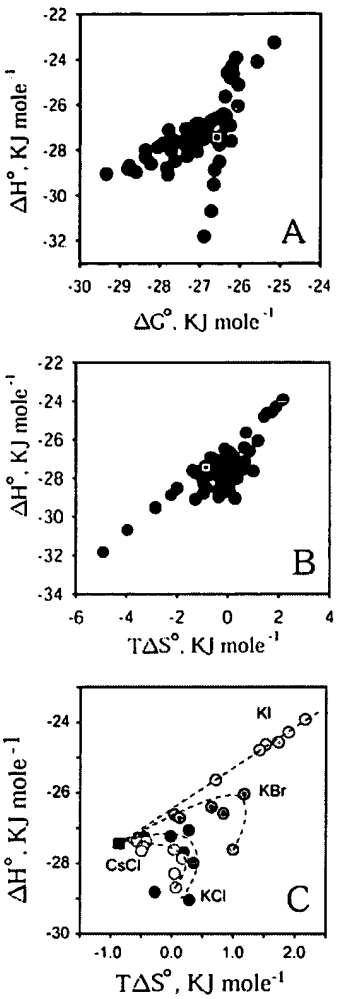


Figure 4. Correlation between (A) heats and free energies of complexation and (B) heats and entropies of association. All cosolutes shown in Figure 3 seem to belong to either of two groups. In blue, KCl, CsCl, LiCl, K_2SO_4, KF, KBr, betaine, and glycine. In red, KSCN, KI, sucrose, and urea. (C) Expanded part of B, showing curved traces in the $\Delta H°-T\Delta S°$ plane for several salts. Colors as for Figure 3; green and red squares correspond to no cosolute addition.

Glycine is most excluded ($\Delta n_w = -24$), while betaine (glycine with a trimethylamine) is most included ($\Delta n_w = +22$). Note that $\Delta G°$ versus osmolal concentration is linear even though betaine and urea preferentially associate with CD/AD, namely, they interact favorably with CD/AD binding surfaces.

In Figure 4, we follow the correlation between the heat release, entropy change, and free energy in different cosolutes. We find that all cosolutes can clearly be associated with one of two cosolute categories, thermodynamically distinct in their mode of action. The first group of cosolutes includes most salts, betaine, and glycine (Figure 4 in blue). Most of the change in binding free energy for these cosolutes is due to a change in heat released upon complexation, while the entropic change is small. Stated in the form $T\Delta S° = \alpha\Delta H° + T\Delta S°_0$, we find an average of $\alpha = 0.39$ for these cosolutes.

The other group of solutes, including KSCN, KI, sucrose, and urea, has smaller Δn_w values for the binding reaction. However, this weak effect on association constant is the result of large, yet compensating changes in the enthalpy and entropy. In this class, $\alpha = 0.91$ with only a small net change in association free energy with cosolute concentration, indicating a small change in cosolute exclusion−inclusion upon CD−AD binding. Such entropy−enthalpy compensations have been previously attributed to processes where water release is believed to be involved[22] and may possibly be related here to a compensation associated with the release of cavity/surface waters.

Figure 4C shows experimental traces in the $\Delta H°-\Delta S°$ plane for KI, KBr, KCl, and CsCl. While we cannot exclude an experimental artifact in determining $\Delta S°$, these cosolutes seem to trace elliptical curves of varying sizes. Such curves have also been reported in experiments by Eftink et al. for protein−ligand binding and cyclodextrin interacting with a series of different guests.[21,56] Interestingly, we find here a similar relationship between entropy and enthalpy of association when solution conditions are varied by added cosolute as when a series of guests of different sizes but similar morphology are used for complexation.

Osmotic Consequences of Molecular Immersion. By measuring the changes in solution osmolality due to addition of macromolecules, it is possible to determine the extent to which a macromolecule preferentially takes up water from the bathing solution, that is, water unavailable to small "excluded" cosolute.[41,52] Assume that every solute macromolecule is surrounded by n_w cosolute-excluding waters. The addition of m_m moles of (macromolecular) solute to 1 Kg of water (≈55.6 mol) will leave only $55.6 - m_m n_w$ water molecules available for dissolution of any other cosolute. Hence, if a solution contains $m_s°$ moles of cosolute, the *change* in the observed osmolal concentration of cosolute due to an addition of m_m solute molecules will be $\Delta m_s^{osm} \cong m_s° m_m n_w/(55.6 - m_m n_w)$. The corresponding *variation* of change in solution osmolality, Δm_s^{osm}, following addition of a small amount of (macromolecular) solute at concentration m_m to solution with initial cosolute concentration $m_s°$, is related to the number of excluding waters per molecule n_w through

$$\frac{d\Delta m_s^{osm}}{dm_s°} \cong \frac{m_m}{55.6} n_w \qquad (3)$$

Equation 3 assumes that m_m is small enough such that intermacromolecule interactions are negligible. Then, from the differences in numbers of excluding waters in reactants and products, we determine the extent of excluding waters released in association.

Once again, due to the link between solute and water chemical potential imposed by the Gibbs−Duhem relation, we can also follow n_s, the excess/deficit number of cosolute rather than n_w, the corresponding number of waters.[39,41] However, we focus our discussion on n_w, which remains constant over the range of cosolute concentrations studied.

The CD/AD complex formed from a 1:1 molar mixture can be considered as *one macromolecular species* because, at the concentrations used, the amount of unassociated CD/AD in the mixtures is less than 5%. From eq 3, it is apparent that changes in osmolalities depend not only on the number of cosolute-excluding waters (n_w) but also on solute concentration (m_m). However, accuracy in measurements of solution osmolality

(56) Eftink, M. R.; Anusiem, A. C.; Biltonen, R. L. *Biochemistry* **1983**, *22*, 3884−3896.

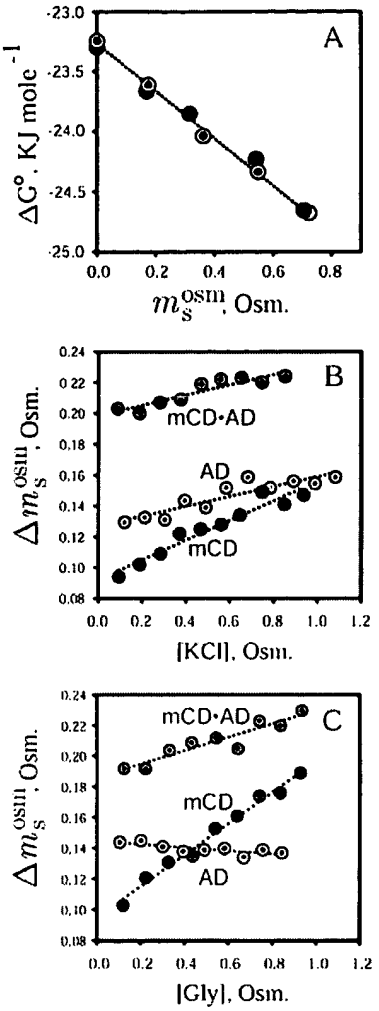


Figure 5. In A, Δn_w is evaluated from changes in association free energies ($\Delta G°$), as determined using ITC, with cosolute osmolal concentration (m_s^{osm}) for mCD showing $\Delta n_w = -40$ for both KCl and glycine. Colors as for Figure 3. In B and C, numbers of cosolute-excluding waters from AD (green), mCD (red), and AD–mCD (magenta), witnessed in the change in solution osmolal concentration (Δm_s^{osm}) with cosolute concentration (eq 3). Cosolutes (B) KCl and (C) glycine. Differences in slopes give changes in cosolute exclusion upon complexation: $\Delta n_w = -43$ in KCl and -31 in glycine.

is limited to ≈2 mOsm, and hence the solubility of β-CD is too low for a precise measurement of osmolality of added cosolute. For this particular measurement, we therefore use a methylated derivative of β-CD, mCD, for which ≈13 hydroxyls (out of 21) per β-CD molecule are randomly methylated. From the correspondence of ITC measurements, performed at low (millimolar) CD/AD concentrations, with osmometry measurements, performed at higher (≈100 mM) concentrations, we conclude that the same association reaction is being probed in both experiments, and competing interactions among CD or AD are negligible.

Figure 5A shows the complexation free energy for mCD/AD in the presence of glycine or KCl at different osmolal concentrations, as derived from ITC. From the slope, we estimate $\Delta n_w = -40 \pm 2$ for both cosolutes. We then immerse small amounts of AD, mCD, or AD–mCD complexes in a cosolute containing bathing solution and measure the effect on solution concentration using vapor pressure osmometry. Figure 5B,C shows the change of measured osmolality upon addition of CD, AD, or an equimolar mixture of the two to a solution of cosolutes: either KCl or glycine. Using eq 3, we derive numbers of excluding waters from the slopes of Δm_s^{osm} with cosolute osmolality.

Though the change in number of excluding waters upon complexation (Δn_w) for KCl and glycine is the same (within experimental error), the number of excluded waters from each of the molecular species is different. Glycine is highly excluded from mCD ($n_w = 61 \pm 4$), but it is almost indifferent toward AD ($n_w = -5 \pm 4$). In contrast, KCl is excluded to a similar extent ($n_w = 36 \pm 4$ and 25 ± 4, respectively) from both mCD and AD. The CD/AD complex excludes KCl and glycine from 18 ± 4 and 25 ± 4 hydrating waters, respectively. The net change for the two cosolutes is $\Delta n_w = -43 \pm 7$ for KCl and $\Delta n_w = -31 \pm 7$ for glycine. Numbers of released waters evaluated using ITC (Figure 5A, $\Delta n_w = -40 \pm 2$) agree reasonably well with those determined from the osmotic pressure measurements. The correspondence in numbers obtained from both ITC and osmometry confirms that both approaches indeed probe the release of cosolute-excluding waters.

It is possible that differences in exclusion of KCl and glycine from the individual CD/AD species are due to differences in interactions of cosolute with surfaces that remain exposed after complex formation (such as CD exterior or the charged carboxylate group on AD), while exclusion from the nonpolar interacting surfaces is similar. Only cosolute that is excluded from interacting surfaces probes water release; exclusion/inclusion from other parts of the molecule is likely unaffected by complexation.

Discussion

Osmotic Stress and Water Release. The osmotic stress technique is becoming an increasingly popular tool for investigating changes in hydration accompanying macromolecular reactions.[28,37,49–51] The approach offers several opportunities. It is widely appreciated that water plays an important role in determining binding energetics, but few methods are available for measuring water release coupled to association. Utilizing osmotic pressure to act on a difference in the number of solute-excluding waters associated with products and reactants is a powerful and practical method for enhancing the stability of complexes. From the dependence of Δn_w on the chemical natures of the probing solute and macromolecular surface, we learn about the nature of the physical interactions between molecules. Last, crowded with salts, sugars, amino acids, and other macromolecules, such as DNA and proteins,[47,57] the intracellular milieu is far different from the dilute aqueous conditions typically used to study the enzymatic, recognition, and assembly reactions that occur in living systems. The osmotic stress technique illustrates how important such "crowding" can be.

By changing the concentration of a cosolute that is excluded from the macromolecule, we evaluate changes in the number of cosolute-excluding waters upon association. Here, we have studied the role of water release in a convenient model system in which two molecules, cyclodextrin (CD) and adamantane carboxylate (AD), present complementary hydrophobic surfaces and associate with specificity.[58]

(57) Yancey, P. H.; Clark, M. E.; Hand, S. C.; Bowlus, R. D.; Somero, G. N. *Science* **1982**, *217*, 1214–1222.

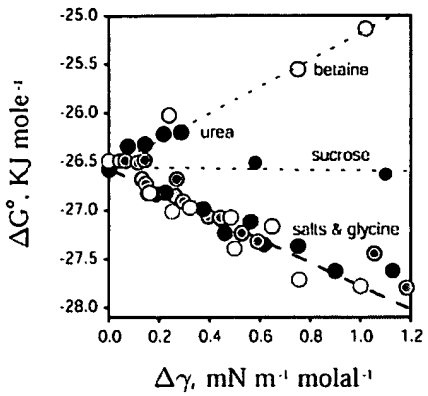


Figure 6. Changes in association free energy correlated with the bathing solution's change in air–water interfacial tension (compared with that of pure water) for different cosolutes. Colors as for Figure 3.

Using ITC, with corroboration from osmometry, we find for many cosolutes that ≈15–25 cosolute-excluding water molecules are released upon complex formation with β-CD. This is consistent with a release of all waters from the cyclodextrin cavity (≈11 waters in the crystal) and additional waters from the adamantane surface.

All cosolutes we have used fall into one of two classes. In the first, which includes many salts and glycine, the contribution to the binding free energy from solute–macromolecule interactions is dominated by enthalpy. For the second group of cosolutes, which are found to have only a weak net inclusion/exclusion, the enthalpic contribution is also large but is almost fully compensated by the change in entropy.

Surface Waters and Surface Tension. One model that has been suggested to account for how cosolutes affect association was first proposed by Sinanoglu and Abdulnur.[59] In that model, cosolute (or solvent) effects were associated with the work needed to create an empty cavity in solution, that is, to surface tensions.[20,40,53,60] To test this idea in CD/AD association, we correlate changes of association strength with surface tensions as measured at the air–water interface for salt and other cosolute solutions.

Figure 6 shows, for most of the cosolutes used in this study, the changes in association free energy as a function of experimentally known changes in air–water interfacial tension.[44,61–65] Strikingly, for many salts, all data fall on a single line. The slope of this line represents an effective surface area change upon complexation of ≈200 Å^2. If we assume a cosolute-excluding water layer one molecule thick (≈3 Å) and a volume of 30 Å^3 per water molecule, we conclude there are ≈20 waters released during the inclusion process. This matches the estimate from the responses to osmotic stress. For KCl and glycine, however, the correlation with surface tension only holds for the difference in waters for the reaction and not for the waters included with the individual species (Figure 5). The correlation seems to reflect only the surfaces that interact in the complex.

Interestingly, the reaction heat capacity for CD/AD complexation was previously found to be −398 J mol^{-1} K^{-1}.[20] Using the range of reported estimates for the heat capacity associated with the burial of exposed hydrophobic surfaces,[16–19] and assuming that CD/AD interacting surfaces are fully hydrophobic in nature, we find a corresponding reduction of 180–340 Å^2 in surface exposed to solution upon complexation. Assuming the existence of a surface layer of cosolute-excluding water 3 Å thick implies a release of 18–34 waters upon complexation. Perhaps fortuitously, this estimate also agrees with the numbers found using the osmotic stress analysis.

For other (predominantly neutral) cosolutes, a correlation does not hold between air–water interfaces and changes in complex stability. We may infer that these cosolutes interact differently with the macromolecule surface than with an air–water interface.

Interactions Involved in Cosolute Exclusion. In terms of the Gibbs adsorption isotherm,[66] the excess or deficit of cosolutes from the interacting surfaces is directly related to changes in surface stability with varied cosolute activity. The excesses or deficits depend on interactions of the solvated cosolute and macromolecule.

We may ask "what interactions are responsible for cosolute exclusion from CD/AD"? Alternatively, we may ask "in what way do surface waters pose a less favorable environment (solvent) to cosolutes"?

One possible origin of exclusion is through steric "excluded volume" interactions between cosolute and CD/AD. Because these forces are manifested due to loss in translational entropy near a macromolecule, no heat evolution is expected. However, experimentally, all cosolutes show a large enthalpic contribution to the change in complexation free energy (Figure 4). While we cannot rule out this as a contributing interaction, we can conclude that crowding due to steric solute–cosolute interactions is not the main contributor to the overall free energy change due to cosolute.

Electrostatic "image charge" interactions of ions with the nonpolar CD/AD surfaces are another possible source of preferential exclusion. By the reasoning of Onsager and Samaras,[67] ions approaching an interface, going from a high to lower dielectric material, are repelled due to loss of favorable interactions with the high dielectric medium. This unfavorable energy competes with the translational entropy of an ion to form an ion-excluded region, typically a few angstroms thick. While the temperature dependence of the original Onsager and Samaras model does not match our findings, and the cyclodextrin cavity and adamantane are not simple nonpolar surfaces, it seems reasonable to expect that such an electrostatic repulsion could contribute to the net exclusion of ions.

What then can account for the differences found among different salts? In particular, the more polarizable ions, such as Br^- and SCN^-, appear here, as in many other experiments, to be less excluded from the macromolecular interface than, say, Cl^- and even more so SO_4^{2-}.[43,44,68–75] Observations of similar

(58) Fu, Y.; Liu, L.; Guo, Q.-X. *J. Inclusion Phenom.* **2002**, *43*, 223–229.
(59) Sinanoglu, O.; Abdulnur, S. *Fed. Proc.* **1965**, *24*, S12–S23 (Supplement 15).
(60) Lin, T. Y.; Timasheff, S. N. *Protein Sci.* **1996**, *5*, 372–381.
(61) Matubayasi, N.; Tsunetomo, K.; Sato, I.; Akizuki, R.; Morishita, T.; Matuzawa, A.; Natsukari, Y. *J. Colloid Interface Sci.* **2001**, *243*, 444–456.
(62) Matubayashi, N.; Miyamoto, H.; Namihira, J.; Yano, K.; Tanaka, T. *J. Colloid Interface Sci.* **2002**, *250*, 431–437.
(63) Washburn, E. W. *International Critical Tables*; McGraw-Hill: New York, 1929; Vol 4 (28).
(64) Soderlund, T.; Zhu, K.; Jutila, A.; Kinnunen, K. J. *Colloids Surf., B* **2002**, *26*, 75–83.
(65) Siskova, M.; Hejtmankova, J.; Bartovska, L. *Collect. Czech. Chem. Commun.* **1985**, *50*, 1629–1635.
(66) Gibbs, J. W. On the Equilibrium of Heterogeneous Substances. In *The Scientific Papers of J. Willard Gibbs*; Bumstead, H. A., van Name, R. G., Eds.; Ox Bow: Woodbridge, CT, 1993; Vol. 1.
(67) Onsager, L.; Samaras, N. N. T. *J. Chem. Phys.* **1934**, *2*, 528–536.

trends go back to the seminal experiments on protein precipitation by different salts that led to the so-called Hofmeister series.[54] It has been proposed that interacting hydrocarbon surfaces (rather than air) introduce the likely van der Waals-type attraction of more polarizable ions to higher index of refraction hydrocarbon.[55,76]

The remarkable correspondence between free energy changes of CD/AD binding due to solute exclusion and surface tensions at air−water interfaces seen in Figure 6 for many of the cosolutes suggests that these cosolutes are interacting with interfacial water that is structured differently from the bulk due to the presence of an air or CD/AD surface. Irrespective of the detailed water structure, this asymmetry will present a locally inhomogeneous dipolar layer of waters to cosolutes. More polarizable ions can "dissolve" better in such a layer, due to an added favorable dipole to induced-dipole interaction.[5,55,77,78] Such an argument could imply a generality of the effect, insensitive to the type of interface formed.[45,53,59] The nature of the surface in contact with water would then be of secondary importance.

Finally, the close interaction of solutes and surfaces necessarily alters the structuring of water around each. The energetics connected with this restructuring of water as two surfaces approach has been suggested as the basis for the common exponential force seen between many macromolecules, both charged and uncharged, at spacings closer than ≈10 Å.[79,80] The extracted spatial dependences for the exclusion of nonpolar alcohols from DNA and of salts and polar solutes from hydroxypropyl cellulose show the same type of exponential behavior as that associated with the postulated hydration force.[81−83] Differences in water structuring around cosolute may, for example, explain the differences in the extent of exclusion/inclusion from CD/AD of glycine with its amine group versus the trimethylated amine analogue, betaine. It is also important to consider water structuring involved in the hydration of cosolutes themselves. Indeed, ions in the Hofmeister series tend to structure water very differently.

Concluding Remarks

Perhaps most important, the results derived using the different approaches are commensurate, enabling a convergence of different perspectives and languages for speaking about interactions of macromolecules in solution. We learn that the solvating power of water around and within cyclodextrin and adamantane differs from that of bulk water, consonant with what has been observed in many specifically interacting biomacromolecules.[84] These waters could be responsible for cosolute exclusion due to their interfacial ordering properties. If so, these waters not only exclude many cosolutes but also preferentially interact with salts to different extents, correlating with ionic polarizability. In concert, direct repulsive solute−surface interactions may also contribute to creating a preferential hydration layer.

By subjecting CD and AD to cosolutes, we show that the strength of their association can be modulated and controlled. Important implications follow because in all technological and pharmacological applications, the systems considered are not pure aqueous solutions, but rather physiological milieus, crowded with small cosolutes and other macromolecules that will affect the binding.[85,86] In fact, cosolute-containing formulations have been proposed as a way to enhance the association of CD with guest molecules.[87] Understanding the preferential interactions of cosolutes with CD will aid in the rational development of effective formulations.

Experimental Section

Cyclodextrins (Fluka) were dried overnight in vacuum; then stock solutions were made by weight. Adamantane carboxylic acid (Fluka) was dissolved in 0.02 M phosphate buffer solution, pH 6.9 (for low concentrations), or titrated with NaOH until fully dissolved, and then buffered in the same way (for high concentration). All chemicals were used with no further purification.

Microcalorimetry. Isothermal titration calorimetric measurements were made at 30 °C using a VP−ITC microcalorimeter (MicroCal). In each experiment, 40 successive 7 μl injections of 5.5−6.5 mM CD solutions were mixed into the thermostated cell containing 0.4−0.5 mM AD solution; solutions were stirred at 300 rpm. Injections lasted 10 s and were spaced at 3.5 min intervals. The heat release associated with AD and CD dilution was measured separately and did not substantially change the calculated thermodynamic properties. Solutions were in 0.02 M Na phosphate buffer at pH 6.9. Both AD and CD solutions contained an additional cosolute, both with the same osmolality.

Equilibrium constants, heats, and entropies of reaction were evaluated using standard MicroCal Origin software procedures. All fits to the data were consistent to within 3% with a 1:1 molar complexation ratio. Attempted fits to other possible complex ratios did not improve the overall fit, supporting the simplest assumption of 1:1 binding under all studied conditions. Presented results are averages of 2−8 repeats and include an uncertainty, expressed as standard deviations, of no more than ±1% for heats of reaction and ±0.5% for the free energies. As detailed in the Results section, changes in numbers of hydrating waters were evaluated from linear least-squares fits to evaluated free energies versus cosolute concentrations. The fits involve an error of, at most, ±5 waters.

Osmolalities of cosolute solutions were measured separately on a Wescor 5520 vapor pressure osmometer.

Osmometry. The osmolality of a series of solutions containing cosolute (KCL or glycine) and water, measured on a Wescor 5520 osmometer, was compared with osmolalities of solutions with the same cosolute molality, but with added solute (AD, CD, or an equimolar mixture of the two). Solute concentrations were in the 70−200 mOsm range and that of cosolute was 50−1000 mM.

Acknowledgment. We thank Horia Petrache for useful comments on the manuscript.

JA045541T

(68) Kotake, Y.; Janzen, E. G. *J. Am. Chem. Soc.* **1989**, *111*, 7319−7323.
(69) Matusi, Y.; Ono, M.; Tokunaga, S. *Bull. Chem. Soc. Jpn.* **1997**, *70*, 535−541.
(70) Leontidis, E. *Curr. Opin. Colloid Interface Sci.* **2002**, *7*, 81−91.
(71) Gelb, R. I.; Schwartz, L. M.; Radeos, M.; Laufer, D. A. *J. Phys. Chem.* **1983**, *87*, 3349−3354.
(72) Yamashoji, Y.; Fujiwara, M.; Matsushita, T.; Tanaka, M. *Chem. Lett.* **1993**, *6*, 1029−1032.
(73) Wojcik, J. F.; Rohrbach, R. P. *J. Phys. Chem.* **1975**, *79*, 2251−2253.
(74) Buvari, A.; Barcza, L. *J. Inclusion Phenom.* **1989**, *7*, 379−389.
(75) Dubois, M.; Zemb, T.; Fuller, N.; Rand, R.; Parsegian, V. *J. Chem. Phys.* **1998**, *108*, 7855−7869.
(76) Bostrom, M.; Williams, D. R. M.; Ninham, B. W. *Langmuir* **2001**, *17*, 4475−4478.
(77) Jungwirth, P.; Tobias, D. J. *J. Phys. Chem. B* **2002**, *106*, 6361−6373.
(78) Garrett, B. C. *Science* **2004**, *303*, 1146−1147.
(79) Leikin, S.; Parsegian, V. A.; Rau, D. C.; Rand, R. P. *Annu. Rev. Phys. Chem.* **1993**, *44*, 369−395.
(80) Rand, R. P.; Das, S.; Parsegian, V. A. *Chem. Scr.* **1985**, *25*, 15−21.
(81) Hultgren, A.; Rau, D. C. *Biochemistry* **2004**, *43*, 8272−8280.
(82) Chick, J.; Mizrahi, S.; Chi, S.; Parsegian, V. A.; Rau, D. C. *J. Phys. Chem. B*, submitted.
(83) Bonnet-Gonnet, C.; Leikin, S.; Chi, S.; Rau, D. C.; Parsegian, V. A. *J. Phys. Chem. B* **2001**, *105*, 1877−1886.
(84) Ball, P. *Nature* **2003**, *423*, 25−26.
(85) Minton, A. P. *Biophys. J.* **2000**, *78*, 101−109.
(86) Miyoshi, D.; Matsumura, S.; Nakano, S.; Sugimoto, N. *J. Am. Chem. Soc.* **2004**, *126*, 165−169.
(87) Li, P.; Zhao, L.; Yalkowsky, S. H. *J. Pharm. Sci.* **1999**, *88*, 1107−1111.